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THE PLASMA PROTEIN CONCENTRATIONS AND ORGAN WEIGHTS OF RATS ON A HIGH PROTEIN DIET[1,2]

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IT HAS BEEN SHOWN that an important relationship exists between the adrenal cortex and the serum protein concentrations. Administration of adrenal cortical extract to hypophysectomized rats will largely prevent the fall in serum albumin usually evident after removal of this gland but does not prevent the rise in globulin concentration. Administration of stilbestrol to stimulate the adrenal cortex of normal rats causes a significant rise in serum albumin concentration (Levin and Leathem, 1942). Moreover, adrenalectomy in cats and dogs where a high degree of adrenal cortical insufficiency is readily obtained is followed by a decrease in serum albumin and a rise in globulin which can be corrected by adequate amounts of adrenal cortical extracts (Levin, Leathem and Crafts, 1942; Hartman, Lewis, Thatcher and Street, 1942). Furthermore, White and Dougherty (1945) have shown that injection of adrenal cortical extract is quickly followed by a rise in serum globulin in rabbits.

Recently, Tepperman, Engel and Long (1943) have reported that a high protein diet causes adrenal hypertrophy in rats and possibly an increased functional level of the cortex as suggested by the increased blood glucose and liver glycogen levels. Adrenal cortical extract has been shown to elevate carbohydrate levels and increase

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nitrogen excretion and thus increases protein catabolism (Long, Katzin and Fry, 1940). Although Ingle, Ginther and Nezamis (1943) and Benua and Howard (1945) failed to observe adrenal hypertrophy following the use of a high protein diet a hyperfunctioning cortex may still have been induced and it appeared of interest to determine whether a high protein diet would influence the albumin-globulin concentrations of the normal rat possibly by increasing adrenal cortical function. Furthermore, the albumin-globulin concentrations of hypophysectomized rats fed either a high protein diet or the stock diet ad libitum were subjects for study in this investigation.

Hypertrophy of the adrenals in animals fed a high protein diet has been reported (Fahr, 1912; Tepperman, Engel and Long, 1943), but Ingle, Ginther and Nezamis (1943) failed to observe this effect in rats. More recently, Benua and Howard (1945) reported their inability to alter the size or histology of male mouse adrenals with a high protein diet. The older literature also suggests that thyroid hypertrophy can be induced with high protein diets (Watson, 1905, 1906; Hunt, 1911; Marine, 1914; Burget, 1917; McCarrison, 1917). The possibility of dietary protein influencing the size of organs prompted the inclusion of organ weights in this study.

MATERIALS AND METHODS

Male rats of the Long-Evans strain and 127 to 140 days old were used in all cases except in group 2. The animals in group 2 were 40 days old at the start of the experiment. Two rats were kept in each metabolism cage for measurement of daily food intake.

The high protein diet used will be found to correspond closely to the casein A diet described by Tepperman et al. (1943). Our diet consisted of 78 per cent casein (Merck), 10 per cent brewers' yeast (Mead Johnson), 5 per cent Wesson's salt mixture, 5 per cent Mazola and 2 per cent cod liver oil (Squibb). The stock diet consisted of purina calf chow plus 10 per cent meat scrap, the latter containing 55 per cent protein. The protein content of the stock diet was approximately 32–33 per cent. Both diets were supplemented by a mixture of two-thirds Mazola and one-third cod liver oil on bread twice weekly and by fresh carrots once each week.

The experimental period extended over a 20 to 24 day period when adult normal or hypophysectomized rats were used and averaged 54 days when the 40 day old rats were tested. Day one of the experimental period corresponded with the time of initial high protein feeding except for a few rats which were fed the high protein diet for 4 days prior to hypophysectomy.

The manner in which blood samples were obtained and the methods of analysis used were the same as those reported previously (Leathem, 1945).

Each rat was autopsied and fresh weight of the carefully dissected pituitary, adrenals, thyroids, testes, seminal vesicles (including the coagulating gland and any contained fluid), ventral prostate, spleen, kidney and liver was taken.

Completeness of hypophysectomy was ascertained by the microscopic examination of the serially sectioned sellas stained with Mallory's connective tissue stain.

RESULTS

Group 1.

Adult rats fed the high protein diet *ad libitum* gained an average of 23 grams in body weight as compared with a 17 gram gain by rats eating the stock diet ad libitum. Average food consumption per rat during the 20-day period was 281 grams when fed the casein diet and 342 grams when fed the stock diet. This voluntary reduction in food intake did not influence the blood proteins since total plasma protein, plasma albumin and plasma globulin concentrations were not significantly changed (Table 1).

TABLE 1. INFLUENCE OF DIETARY PROTEIN ON THE PLASMA PROTEIN LEVELS OF MALE RATS

Rats (no.)	Diet	Days fed	Body weight start-end	Hemato-crit	Non-protein nitrogen	Total protein	Albumin	Globulin	a/g ratio
			gm.	per cent	mg./100 cc.	gm./100 cc.	gm./100 cc.	gm./100 cc.	
Group 1									
Normal (13)	HP	20–24	241–264	46.6±1.5*	59.8±3.1	6.43±0.18	3.98±0.17	2.44±0.12	1.75±0.16
Normal (20)	S	20–24	235–252	45.4±0.7	53.5±0.8	6.37±0.07	3.86±0.09	2.51±0.10	1.57±0.10
Group 2									
Normal (9)	HP	54	90–247	49.9±1.1	81.3±4.7	6.40±0.17	4.20±0.13	2.17±0.32	1.98±0.08
Normal (9)	S	54	94–246	46.8±1.1	53.7±3.7	6.36±0.12	4.06±0.10	2.30±0.12	1.73±0.13
Group 3									
Normal (10)	HP	20	282–300	48.7±0.4	79.0±4.3	6.24±0.12	3.88±0.14	2.36±0.13	1.83±0.17
Normal (10)	S	20	290–270	47.7±2.4	70.4±7.3	6.07±0.16	3.61±0.10	2.45±0.11	1.51±0.10
Group 4									
Hypox (21)	HP	20	266–191	46.3±1.0	88.0±6.8	6.25±0.28	3.12±0.14	3.13±0.19	1.00±0.08
Hypox (13)	S	20	267–194	41.8±1.4	70.0±4.4	6.28±0.24	3.13±0.18	3.15±0.16	1.02±0.06

HP = high protein diet.
S = stock diet.

* mean deviation of mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

A significant increase in adrenal weight was not observed. In fact the only organ definitely influenced, of those examined, was the kidney. A significant increase in kidney weight was induced (Table 2). Only the actual weights of the organs are presented since body weights differ only slightly.

Group 2.

Failure to obtain adrenal hypertrophy in adult rats fed the casein diet for a 3 week period prompted the study of young rats and an extension of the feeding period. For these experiments 40 day old rats were fed a high protein diet for 38 to 69 days (average 54). During the first 10 days body weight increase was retarded in comparison with littermate controls eating the stock diet ad libitum. Food con-

sumption of rats on the casein diet, however, was markedly reduced, but food consumption increased rapidly about the tenth day and body weight levels were very quickly equal to those of the stock diet controls. Final body weights in the two groups were virtually identi-

TABLE 2. INFLUENCE OF DIET ON ORGAN WEIGHTS OF NORMAL AND HYPOPHYSECTOMIZED RATS

	Average organ weight gr.			
	Normal		Hypophysectomized	
Diet (ad lib) (No. of rats)	Stock (18)	High Protein (13)	Stock (13)	High Protein (19)
Pituitary	0.008±0.0003*	0.008±0.0002	—	—
Adrenal	0.024±0.002	0.028±0.002	0.007±0.0004	0.008±0.0004
Thyroid	0.020±0.0009	0.018±0.0008	0.012±0.0003	0.012±0.0005
Testes	2.715±0.077	2.621±0.128	0.746±0.048	0.924±0.042
Seminal vesicles	0.891±0.115	0.880±0.092	0.134±0.007	0.155±0.007
Ventral prostate	0.248±0.024	0.227±0.024	0.031±0.003	0.034±0.004
Spleen	0.973±0.088	1.026±0.086	0.672±0.053	0.654±0.015
Kidney	2.221±0.102	2.835±0.110	1.358±0.070	1.577±0.064
Liver	10.026±0.441	11.444±0.503	5.919±0.310	6.566±0.277

* mean deviation of mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

cal (Table 1). No significant change in the plasma proteins was evident in spite of the longer experimental period but non-protein

TABLE 3. INFLUENCE OF DIET ON ORGAN WEIGHTS IN 40 DAY OLD RATS

	Average organ weight gr.	
Diet (ad lib) (No. of rats)	Stock (10)	High Protein (10)
Pituitary	0.007±0.0004*	0.007±0.0004
Adrenal	0.028±0.002	0.029±0.002
Thyroid	0.020±0.003	0.018±0.0009
Testes	2.789±0.109	2.679±0.107
Seminal vesicles	0.709±0.074	0.660±0.080
Ventral prostate	0.164±0.019	0.178±0.018
Spleen	0.664±0.057	0.700±0.070
Kidney	2.392±0.117	3.106±0.182
Liver	10.534±0.605	12.310±0.630

* mean deviation of mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

nitrogen was significantly higher in the plasma of rats fed the 78 per cent casein diet than in rats on the stock diet (Table 1).

Adrenal weight was not increased. Furthermore the weights of the other organs were not influenced with the exception of the kidney and possibly the liver. Renal hypertrophy was quite marked in this group. Liver weights were extremely variable and the difference in average weights is of questionable significance (Table 3).

Group 3.

A voluntary reduction in food consumption was apparent when rats were placed on a high protein diet. The average reduction in food intake per rat per day for 20 days was 3 grams. What effect this reduced food intake would have on rats fed the stock diet was tested in this group by paired feeding. Food consumption of each rat on the high protein diet over the 20 day period was 280 grams and the average increase in body weight was 18 grams. These results compare favorably with those obtained in Group 1. Restricting the food intake of rats on the stock diet to that of the daily consumption by rats on the casein diet resulted in an average body weight loss of 20 grams. Examination of the blood proteins revealed the trend usually observed when food intake is restricted, that is, a reduction in total protein and plasma albumin concentration (Table 1).

An increase in kidney and liver weights were observed in rats on the high protein diet. The kidneys of rats fed the casein diet were 1071 grams/100 grams body weight as compared with kidney weight of 910 grams/100 grams body weight of rats on stock diet. Liver weights in grams/100 grams body weight were 4563 and 3520 for rats on high protein and stock diet respectively. No other organ weight changes of significance were noted.

Group 4.

Hypophysectomized rats were studied during the first 20 days following the operation and total food consumption per rat on stock diet was found to average 135 grams. This represented a marked reduction in food intake since normal rats consume 342 grams in the same time period. A still greater reduction in food intake by hypophysectomized rats was observed when the high protein diet was used, the animals eating only 93 grams total for 20 days. Body weight loss normally observed after hypophysectomy was not altered by the high protein diet.

Ablation of the pituitary was followed by only a slight decrease in total plasma protein but plasma albumin was decreased markedly, being only 3.13 grams per cent as compared with 3.86 grams per cent in normal rats. Moreover, plasma globulin increased from 2.51 grams per cent in normal rats to 3.15 grams per cent in hypophysectomized rats. The 78 per cent casein diet failed to modify the plasma protein changes observed after hypophysectomy (Table 1).

Organ atrophy typically observed following hypophysectomy was not significantly influenced by the high protein diet.

DISCUSSION

Hypertrophy of the adrenal cortex due to a high protein diet has been reported to occur in rabbits (Fahr, 1912) and in rats (Tepperman, Engel and Long, 1943), but other investigators have failed to

observe this effect (Ingle, Ginther and Nezamis, 1943; Benua and Howard, 1945). Adrenal hypertrophy was not obtained in our experiments in which the casein A diet of Tepperman, Engel and Long (1943) was used and which for these investigators had resulted in a 40 per cent adrenal weight increase. Factors other than those which are apparent at the moment must be responsible for the difference in results.

Failure to observe adrenal hypertrophy does not necessarily mean that the functional level or the balance of steroids produced by the cortex has not been altered. Therefore, the effect of a high protein diet on the albumin-globulin concentrations was investigated since the adrenal cortex is concerned with protein catabolism (Long, Katzin and Fry, 1940) and appears to be concerned with the maintenance of a normal albumin concentration (Levin and Leathem, 1942; Levin, Leathem and Crafts, 1942; Hartman, Lewis, Thatcher and Street, 1942) as well as being capable of increasing serum globulin (White and Dougherty, 1945). However, plasma proteins were not significantly altered in normal rats fed a high protein diet ad libitum. Bloomfield (1935) observed a decrease in total blood protein when rats were fed 100 per cent casein for 20–40 days, but normal blood levels were regained after 100 days on this diet. Since body weight loss was rather marked at the time when total protein levels were decreased it would appear that the initial drop might be due to a marked reduction in food intake. In our experiments extending over a 20 day period, total protein levels were normal in the rats fed 78 per cent casein.

Rats on a high protein diet voluntarily reduced their food intake an average of 62 grams over the 20 day period. Limiting the food intake of the stock diet by paired feeding to that of casein fed rats resulted in a decrease in total plasma protein due to a decrease in plasma albumin. It is known that inanition produces alterations in serum protein levels (Weech, 1938), and a low protein diet quickly lowers total serum protein in the rat, the decrease being due to a drop in serum albumin concentration (Bloomfield, 1933).

Hypophysectomy is followed by a drop in serum albumin and a rise in globulin concentration which has been shown to be only partially due to inanition (Levin and Leathem, 1942; Levin, 1943; Moore, Levin and Leathem, 1944). Our experiments show that the albumin-globulin concentrations of the hypophysectomized rat eating ad libitum are not improved by a 78 per cent casein diet.

The high protein diet did not retard body weight gain by normal animals fed ad libitum except for a short initial period with the 40 day old rats. Bloomfield (1935) however, observed a marked decrease in body weight of rats fed 100 per cent casein for 20–40 days. Although the rats regained some of the lost weight after 100 days, they were still 10 per cent underweight at autopsy. Some effects of a high protein diet have also been noted in experiments involving bilateral

or unilateral adrenalectomy. For example, Ingle, et al. (1943) observed that adrenalectomized rats maintained on a 1.0 per cent sodium chloride solution exhibited greater body weight gains on a high protein diet than on other diets. On the other hand, Benua and Howard (1945) found a retardation of body weight increase in unilaterally adrenalectomized mice on an 89 per cent protein diet as compared with those on a 16 per cent protein diet.

It has been reported that thyroid hypertrophy can be induced by a high protein diet (Watson, 1905, 1906; Hunt, 1911; Marine, 1914; Burget, 1917; McCarrison, 1917). No evidence of thyroid hypertrophy was obtained in our experiments with the 78 per cent casein diet and no indication that a hypothyroid state was developed since the globulin concentration which increases in hypothyroidism (Levin and Leathem, 1942; Leathem, 1945; Moore, Levin and Smelser, 1945), remained unchanged.

The weight of the pituitary, testes, seminal vesicles, ventral prostate and spleen remained within normal range despite the high protein diet but a significant renal hypertrophy was evident. The increase in kidney weight was most apparent in the young rats maintained on the casein diet for the average 54 day period. Reid (1944) induced kidney hypertrophy with a protein diet of lean meat only and found that the enlarged kidneys contained the same percentage of total nitrogen as normal kidneys. The renal hypertrophy was not observed in the absence of the pituitary by Reid (1944) nor in our experiments. Levin (1944) has shown that post-hypophysectomy weight loss of the kidney was not prevented by force feeding although these rats gained in body weight. Average liver weight was greater in rats on a high protein diet but a significant difference was observed in only one group.

SUMMARY

Plasma albumin-globulin concentrations were not significantly altered in normal or hypophysectomized rats fed a high protein diet ad libitum in comparison with rats on stock diet. A voluntary reduction in food consumption resulted with a high protein diet. This reduction was sufficient to reduce total protein levels in pair fed rats on stock diet, due to a decrease in plasma albumin concentration. Marked changes in albumin-globulin concentrations were evident following hypophysectomy irrespective of diet.

Normal rats on a high protein diet gained in body weight at the same rate as rats on the stock diet when ad libitum feeding was permitted.

A 78 per cent casein diet did not cause adrenal hypertrophy nor was the weight of the pituitary, thyroid, testes, seminal vesicles, ventral prostate or spleen altered. This high protein diet, however, caused a definite renal hypertrophy in normal rats but was without effect in hypophysectomized rats.

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THE EFFECTIVENESS OF GONADOTROPIN INJECTIONS FOLLOWED BY INSEMINATION IN INDUCING PREGNANCY IN EWES[1]

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OVULATION following the injection of gonadotropins into ewes has been reported by various investigators to be rather consistent (Cole and Miller, 1935; Zawadowsky, Vunder, Padoocheva, and Margvelashvilla, 1935; Cole, 1936; Loginova, 1939; Bell, Casida, Bohstedt, and Darlow, 1941; Hammond, Hammond, and Parkes, 1942; Frank and Appleby, 1943). That at least a part of the ova produced by such treatment are fertile is suggested by the fact that fertilization and pregnancies have been recorded in several instances (Zawadowsky, et al., 1935; Cole and Miller, 1935; Casida, Warwick, and Meyer, 1944).

Behavioral heat in the ewe has not resulted usually from single injections of gonadotropins and as a result relatively few animals have been bred following treatment. Zawadowsky et al., (1935) and Loginova (1939) reported that pregnancy was obtained by artifically inseminating ewes after injection of gonadotropin from pituitary or from urine of pregnant women. Attempts by Polovtzova and Judovitch (1939) and Frank and Appleby (1943) to induce pregnancy following single gonadotropin injections were less fruitful.

A group of ewes on experiments at this Station have been used to investigate the effectiveness of gonadotropin injections followed by insemination in inducing pregnancy, with special reference to the time interval between injection and insemination, to the condition of the genital tract of the ewe at time of breeding, and to the effect of estrogen injections in an attempt to induce uterine conditions simulating those found in natural estrus.

PROCEDURE AND RESULTS

Preliminary observation: In the spring of 1943 eight yearling and four mature Rambouillet ewes were checked daily for heat from February 13 to April 29. During this time one mature ewe was in heat on April 28. This observation is in agreement with previous findings at this Station (Bell, 1945). On March 1, two of the anestrus yearling

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ewes were each given 5.0 cc. (250 R.U.) of pregnant mare serum[3] subcutaneously, and force-bred two days later. There were no apparent signs of estrus shown by either ewe during the period of observation. One of the ewes gave birth to a normal lamb on July 27.

First year's work with ewes during the normal breeding season: A group of 110 yearling grade Rambouillet ewes was observed for heat from October 12 to November 16 in order to determine the normal cycle of the individual animals. Ewes that did not show natural estrus with a normal length cycle were excluded from the study. On November 17, 36 ewes were injected with 5.0 cc. of pregnant mare serum and 36 with 3.0 cc. of crude pituitary extract representing 36 gm. of fresh anterior lobe. Each group of 36 was divided into four subgroups of nine animals each, in various stages of the estrual cycle. One subgroup from each treatment was inseminated on the first, second, third, and fourth day respectively following hormone injection.

Seven ewes lambed as a result of the above treatment. Two ewes which lambed were inseminated near the time of estrus and fertilization could have been a result of natural processes. This left a total of five ewes that lambed almost certainly as a result of insemination following hormonally induced ovulation (table 1, numbers 20, 5, 26, 7, and 77). Three pregnancies resulted from insemination on the second day after injection of pregnant mare serum. Two of the ewes lambed as a result of insemination following the injection of pituitary extract, one having been inseminated on the second day and one on the third day following treatment.

The pregnancies resulted from treatment at various stages of the cycle with no marked indication that treatment during one period of the cycle was more effective than at any other time.

There was one observation that was suggestive of significance in the results obtained. All pregnancies occurred in ewes which at the time of insemination exhibited a thin vaginal mucus similar to that of ewes in natural heat. Experience has shown that this condition of the vaginal mucus is rarely observed in untreated ewes except at the time of natural estrus. The history of the ewes which were considered to lamb as a result of induced ovulation indicate that they were not in natural estrus at the time of insemination (table 1). With the exception of one ewe which failed to show heat after breeding, the ewes were inseminated between natural estrual periods of 15 to 18 days apart. Furthermore, none of the ewes were receptive to the ram at or near to the time of insemination.

Of the 36 ewes examined on the second or third day following gonadotropin injection, 12 which were not receptive to the ram within two days of examination showed a thin vaginal mucus and

[3] "Gonadin" from the Cutter Laboratories, Berkeley, California.

apparently open cervix. Five of this number or approximately 42 per cent became pregnant.

Second year: In view of the observation that in most of the ewes the cervix remained closed by a thick mucus following gonadotropin injections, it appeared advisable to attempt to condition the reproductive tract to more nearly simulate that found in natural estrus. Estrogen appeared to be the logical agent to use since it had been shown by Bell et al. (1941) to cause a thinning of vaginal mucus. Estrogens have been observed also to cause ovulation in sheep under

TABLE 1. TIME OF HORMONE INJECTION AND BREEDING IN RELATION TO THE ESTRUAL CYCLE OF EWES THAT LAMBED AS A RESULT OF INDUCED OVULATION AND INSEMINATION

Ewe number	Date of first day of cycle[1]	Day of cycle																	Date ewe lambed[2]
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
20	11-8-43	E						P*		B							E			4-13-44
5	11-12-43	E		P	*	B													E?	4-14-44
26	11-9-43	E					Pt	*	B									E		4-14-44
7	11-3-43	E											P		B*			E	E	4-15-44
77	11-8-43	E						Pt		*		B						E		4-15-44
63	10-29-44	E		P		B*	B										E			3-27-45
2	10-22-44	E						PS		B*		B					E			3-29-45
13	10-22-44	E								PS		B*	B					E		3-31-45
49	10-30-44	E	PS		B	B*														4-1-45
35	10-20-44	E											P	*	B	B		E?	E	4-2-45

E signifies estrus; P, 5.0 cc. P.M.S. subcutaneous; S, 5.0 mg. stilbestrol in oil given subcutaneously; B, ewe bred or inseminated; Pt, 3.0 cc. pituitary extract.

* 150 days previous to lambing.

[1] All ewes had also shown normal estrus 15 to 18 days previous to date indicated.

[2] Estrus did not occur between the last time shown in the table and time of lambing.

certain conditions (August, 1941; Hammond, Hammond, and Parkes, 1942; Guin and Van der Wath, 1943) although an antagonistic response to gonadotropin was reported by Cole and Miller (1935) and by Frank and Appleby (1943). However, ovulation has been observed following simultaneous injection of gonadotropin and various estrogens (Hammond, Hammond, and Parkes, 1942; Frank and Appleby, 1943).

Sixty ewes which showed normal estrual cycles were divided into four groups of 15 each. One group served as negative controls, received no hormone injection, but were inseminated along with those receiving treatment. The remaining three groups received subcutaneous injection of 5.0 cc. pregnant mare serum, 5.0 mg. of stilbestrol in oil, and 5.0 cc. pregnant mare serum plus 5 mg. stilbestrol, respectively. One ewe from each group (except negative controls) was injected on each day of the estrual cycle through the fifteenth day, counting the last day of heat as the first day of the cycle. All ewes were inseminated on both the second and third days following treatment.

The appearance of the vaginal mucus following treatment and the lambs born in the different groups are summarized in table 2.

The results from giving pregnant mare serum were similar to those of the previous year. Three lambs were born from this group, two of them being considered due to fertilization after induced ovulation (numbers 35 and 63, table 1). Five mg. of stilbestrol caused a thinning of the mucus apparently in four ewes that were injected at a time when natural estrus was not expected. Only one ewe was receptive to the ram, although several exhibited behavior approach-

TABLE 2. LAMBING RECORD, RECEPTIVITY, AND VAGINAL SECRETION OF EWES INJECTED ON DIFFERENT DAYS OF THE ESTRUAL CYCLE

Hormone treatment	Day of cycle injection was given and appearance of vaginal secretion on second and third days after injection**															Total number of lambs born	Number of lambs from induced ovulation
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
Controls, no injection	M	T	T	T	T	T	T	T	T	T	T	T	T	M*[1]	F*[1]	2	0
5.0 mg. stilbestrol	T	M	M	T	T	T	T	F*	T	M	T	T	T	M*	F*[1]	1	0
5.0 cc. (250 R.U.) P.M.S.	T	T	F[1]	M	T	T	M	T	F	T	F	F[1]	T	T	F*[1]	3	2
Stilbestrol and P.M.S.	T	F[1]	M	T	F*	T	M[1]	M	F[1]	T	M	F*	T	F*[1]	F*[1]	5	3

[1] Ewe lambed 148–151 days after insemination.
* Ewe receptive to ram.
** T signifies thick or cheesy mucus; M, medium; and F, thin or fluid mucus. When there was a difference in appearance on successive days, the thinner classification was used.

ing that of ewes in heat. One lamb was born from the stilbestrol-treated group but was likely a result of natural ovulation (table 2, fifteenth day).

In the group receiving simultaneous injections of pregnant mare serum and stilbestrol, two of the ewes were inseminated at or near the time of estrus. Of the remaining 13 ewes, eight had vaginal mucus showing some similarity to that of ewes in natural estrus. None of the ewes in the control group showed this condition except those which were examined near the time of estrus. Two of the ewes given simultaneous injections became receptive to the ram apparently due to treatment but neither of them became pregnant. Five ewes in the group lambed, three of them as a result of induced ovulation (numbers 13, 49, 35, table 1).

All of the ewes in the control group except those inseminated within one day of estrus showed a thick or cheesy vaginal secretion. Only two lambs were born in this group and these were to ewes which were in heat at the time of insemination.

DISCUSSION

The reports mentioned in the introduction leave little doubt that ovulation can be induced in sheep by means of gonadotropic hormones. Where detailed studies have been made, the percentage of ewes which ovulated due to gonadotropic treatment has been high

in most reports although data on the response to treatment during different periods of the reproductive cycle are incomplete.

While this study gives no indication of the total percent of ewes ovulating due to treatment, it appears that ewes in different periods of the estrual cycle during the regular breeding period ovulated due to gonadotropin injections. A study of the case histories of the individual animals leaves little doubt concerning this point (table 1).

Factors other than ovulation have a bearing on inducing pregnancy by artificial means. The observation that the cervix remained closed in a majority of the cases following injection suggests that failure of the sperm to traverse the cervix may be one item. This factor was also suggested by Polovtzova and Judovitch (1939). The attempt to remove this obstacle by use of estrogen resulted in a slight increase in rate of conception but the rate was still low and numbers too small to be considered significant. The timing of insemination is also a factor, but the first year's results indicate that there would have been little advantage in inseminating other than on the second and third days. There are other considerations such as infertility of ova and failure to implant, on which there is little evidence and which are in need of clarification by more detailed experimentation.

The fact that the rhythm of the natural cycle was not disturbed by injection of either gonadotropin or stilbestrol is of interest. With the exception of one ewe bred early in the cycle the ewes that conceived also returned in heat at the time of expected estrus. Another of the ewes bred early in the cycle while showing indications of estrus was of doubtful receptivity. None of the ewes which became pregnant came in heat more than one time following conception.

There were no apparent harmful after effects from any of the hormone treatments given. All but three of the ewes which did not conceive during the experimental period were later bred at natural estrus and settled. These three exceptions are no more than usual in similar untreated ewes.

SUMMARY

One of two ewes which were injected with pregnant mare serum during the anestrus period and force-bred 2 days later lambed as a result of treatment.

Seven of 46 ewes which were injected with gonadotropin from P.M.S. or pituitary extract during the regular breeding season and inseminated on the second and/or third day following, lambed as a result of induced ovulation. Ewes which were inseminated near time of natural estrus were not counted. All the pregnancies occurred in 16 ewes which exhibited vaginal secretions at time of insemination similar to that observed in natural estrus.

Four of 14 ewes injected with 5.0 mg. stilbestrol when estrus was not expected showed vaginal mucus partially similar to that of ewes

in heat. One was receptive to the ram but none conceived from insemination on second and third days.

Eight of 14 ewes given simultaneous injections of P.M.S. and stilbestrol when estrus was not expected showed a thin vaginal mucus and three of these conceived to insemination on the second and third days after injection.

The natural rhythm of the cycle was not altered by hormone treatment given and no harmful after effects were observed.

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A STUDY OF HYALURONIDASE-EFFECTS ON THE FOLLICLE CELLS OF OVULATED RAT OVA[1]

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HYALURONIDASE, an enzyme which is prepared from mammalian testis, depolymerizes the muco-polysaccharide hyaluronic acid and lowers the viscosity of substances containing hyaluronic acid (Ann. Rev. Biochem, 1944). It is also capable of dispersing the follicle cells which surround recently ovulated mammalian ova (McClean and Rowlands, 1942; Fekete and Duran-Reynals, 1943). The suggestion was made by the latter workers that this enzyme, arising from the numerous spermatozoa, is instrumental in dispersing the follicle cells so that fertilization by one sperm is readily accomplished. In view of these findings, an investigation was begun on the possible role of hyaluronidase in fertility in mammals. The first experiments were to determine if immunization of animals to hyaluronidase would prevent the follicle cells from being dispersed in the presence of the enzyme and if the antiserum would inhibit the action of the enzyme *in vitro*.

METHODS

Two preparations of hyaluronidase from bull testes were used, one prepared by Dr. E. Schwenk[2] and a bull testis extract made in this laboratory by the method of Aylward (1937). Solutions of the dried powder were prepared approximately 1 hour before using by dissolving 1 mg. in 2.5–5 cc. of Ringer's or Locke's solution.

Rat ova were secured in a few cases by hormonally induced ovulation in immature rats (Rowlands, 1942) or in most cases by breeding adult females to vasectomized bucks in the evening and removing the tubal ova the following morning. The ova were washed in Locke's or Ringer's solution and transferred with a fine glass pipette to a concave depression slide so that they were in .15–.20 cc. of fluid. .05–.10 cc. of hyaluronidase solution were added and the contents of the slide stirred well with a needle. The slides were placed in petri dishes containing filter paper moistened in order to minimize evaporation. All tests were conducted at room temperature.

Five adult female rats were injected intraperitoneally with 2 cc. of the bull testis extract on alternate days for 2 months. Beginning one week after

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[2] We are indebted to Dr. Irwin Schwenk of the Schering Corporation, Bloomfield, N. J., for his preparation of hyaluronidase.

the last injection, the rats were bred as they came into heat to vasectomized males and the ova obtained. The blood was secured by bleeding from the heart. The serum was added to the ova first and the enzyme stirred in immediately afterwards.

Observations were made at regular intervals on the degree and rate of dispersal of the follicle cells with the aid of a binocular dissecting microscope. In all, 123 tests were made.

RESULTS

Preliminary experiments were made to determine the optimum amount of enzyme needed to disperse the follicle cells at a rate and to a degree that the end-point could be readily determined. This amount was found to be .01–.05 mg. of dried extract when the ova were suspended in Ringer's or Locke's fluid. The ova, usually all in a group, separated and lost almost all of their follicle cells in a period of 1 hour. Some ova will be completely denuded (designated +++); some will be partially denuded so that a definite area of the zona pellucida is freely exposed (++); some will be covered by what appears to be a single uniform layer of follicle cells (+). Those ova not affected are noted "unchanged" (0). The follicle cells dispersed as in categories ++ and + could be easily removed by gently moving the ova with a needle, so for practical purposes it was considered a positive result. When the enzyme was ineffective, it was seemingly impossible to remove the follicle cells from the ova by moving them with a needle or by even more rigorous physical treatment.

It was found that the follicle cells of the ova from 5 immunized rats were as readily dispersed by hyaluronidase as the follicle cells from non-immunized rats. The ova were suspended in salt solution and all tests were made in duplicate, using ova from each oviduct. Duplicate controls were run simultaneously and within one hour, the follicle cells in both groups were dispersed to the same degree.

Difficulties arose when tests were made on the effect of blood serum on this reaction. It was discovered that normal rat serum (from either sex) prevented the action of the enzyme in doses which would adequately disperse the follicle cells within 1 hour if they were in Ringer's solution (10 cases with 10 controls). In 2 cases when serum was added, the follicle cells were unchanged for 20 hours, but upon washing the eggs with Ringer's and adding enzyme at the same level, the follicle cells were dispersed in 1 hour.

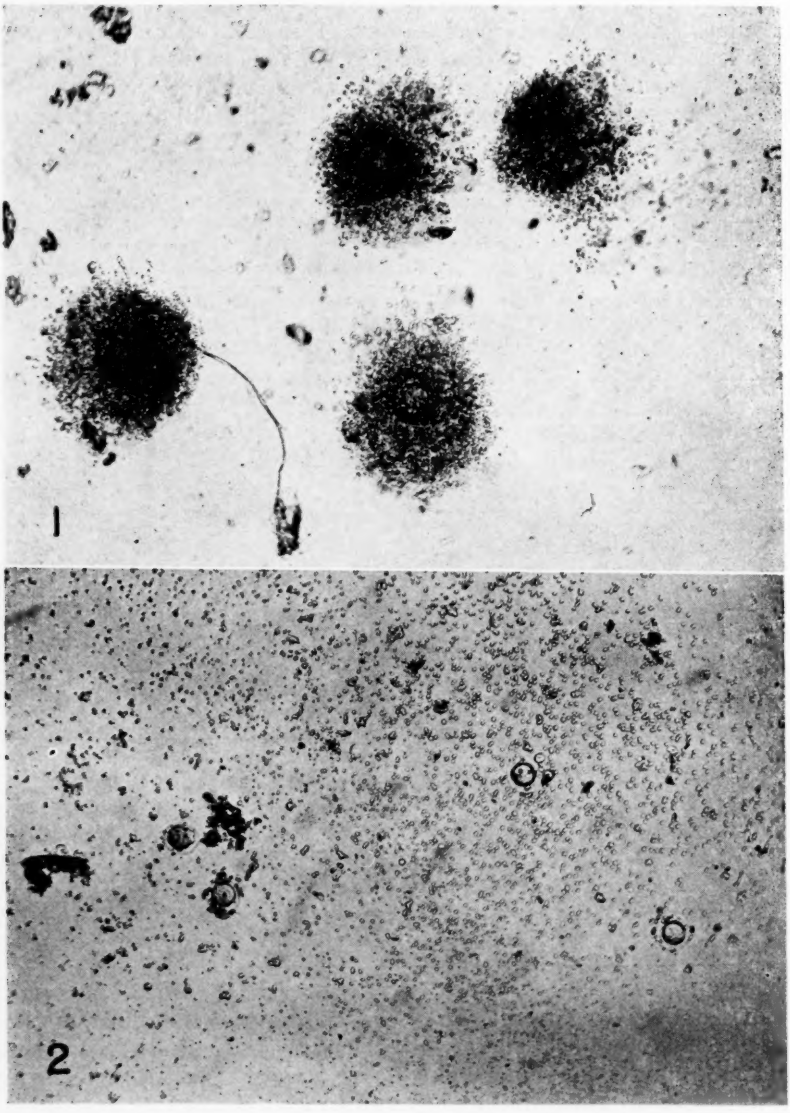
Normal serum was then diluted 1:1, 1:5, 1:10, 1:100 with Ringer's and the effect of the enzyme studied. It was found that normal serum diluted 1:1 completely inhibited hyaluronidase, 1:5 inhibited the reaction very greatly (denuded ova in 20 hours), 1:10 inhibited the reaction only slightly (positive effect 1½–2 hours) and dilution 1:100 had no inhibitory effect. In order to differentiate the inhibitory action of normal serum from that of antiserum all subsequent tests were made at a dilution of 1:10.

The effects of the antisera of 5 rats on the dispersing action of hyaluronidase upon normal rat ova are given in Table 1. All tests were made in duplicate, comparing the effects on the ova from the same rat. It was noted that in every case, either the antisera inhibited the reaction completely or the rate of reaction was inhibited. Figures 1 and 2 illustrate the effect of these sera on the follicle cells.

TABLE 1. EFFECT OF NORMAL AND ANTI-HYALURONIDASE SERUM ON HYALURONIDASE ACTIVITY ON OVA, MEASURED IN TIME AND DEGREE OF ACTIVITY

Treatment	Time in Hours for Hyaluronidase Reaction			
	Degree of Activity			
	Denuded +++	Partially covered ++	Uniform layer +	Unchanged 0
	(hours)	(hours)	(hours)	(hours)
Antiserum #3				3
Normal serum	3			
Antiserum #3				4
Normal serum	4			
Antiserum #5				8
Normal serum			1	
Antiserum #5				
Normal serum		2½		8
Antiserum #6				2
Normal serum	2			
Antiserum #6				7½
Normal serum	2½			
Antiserum #9				8
Normal serum	4¾			
Antiserum #9				4
Normal serum	4			
Antiserum #10		4½		
Normal serum		1		
Antiserum #10	4			
Normal serum	1¾			

Belatedly, two attempts were made to study the effect of the uterine fluid, removed at the time the eggs were obtained from the immunized rats, on the action of the enzyme. For controls, the uterine fluid from non-injected rats was used. The uterine fluid from one of the two immunized rats inhibited the enzyme, (duplicate tests), but the fluid from the other rat was without effect. It should be added that precipitin tests (ring test) were negative for the serum in the rat whose uterine fluid failed to inhibit the action of the enzyme but were positive in the rat whose uterine fluid inhibited the action of the enzyme.



EXPLANATION OF FIGURES

FIG. 1. Ova with follicle cells unchanged. The ova were suspended in .15 cc. of antiserum #9 diluted 1:10 plus .10 cc. of hyaluronidase solution. Photograph taken 4 hours later.

FIG. 2. Ova from the same rat as above, showing complete dispersal of follicle cells. The ova were suspended in .15 cc. of normal female rat serum diluted 1:10 plus .10 cc. of hyaluronidase solution. Photograph taken 4 hours later.

DISCUSSION

These results definitely indicate that antisera formed against bull testis extracts containing hyaluronidase can inhibit or prevent its action in dispersing the follicle cells of rat ova *in vitro*. On the other hand, ova from these immunized rats were affected by hyaluronidase at the same rate as ova from normal rats. Previous work on antibody formation against this enzyme and its activity has been reported in which a different type of test was employed (McClean, 1943). The above findings agree in principle with his results.

In view of these experiments, suggestions of the use of hyaluronidase in problems of artificial insemination are obvious. One of us (R. K.) has used hyaluronidase in 5 cases of artificial insemination in the human, employing previously tested potent donors. The semen specimens during previous months failed to produce pregnancies but did so promptly upon the addition of 20 mg. of hyaluronidase prepared from bull testis.

The observation that normal rat sera will inhibit hyaluronidase reaction on the follicle cells has its counterpart in clinical observations which might account for some forms of human sterility. Bleeding which is occasionally produced during dilation of the cervix at the time of artificial insemination is detrimental to fertilization. Also clinical experience had shown that with normal coitus, patients frequently fail to conceive when bleeding from lesions of the cervix or uterus.

Further investigation might also reveal that some types of sterility, previously attributed to sperm immunity may be due to antibodies formed against hyaluronidase which is presumably associated with the sperm. Antibodies could pass from the serum into the uterine fluid and prevent fertilization at the optimum time as was suggested by one of the above experiments with rats. Investigations are now being conducted to determine whether the blood serum of sterile women, in whom no demonstrable cause for sterility can be shown, has an inhibitory effect upon the hyaluronidase reaction on rat ova.

SUMMARY

The recent discovery that the enzyme hyaluronidase prepared from mammalian testis is capable of dispersing the follicle cells surrounding recently ovulated mammalian ova has been confirmed. It was found that the follicle cells of ova from rats which were immunized against bull testis extracts (containing hyaluronidase) were dispersed as readily as those from normal rats. Normal undiluted rat serum inhibited the action of the enzyme on the follicle cells *in vitro* but sufficiently diluted serum would not inhibit the reaction. Diluted antisera of rats immunized against hyaluronidase inhibited the action of the enzyme on the follicle cells *in vitro* whereas normal rat serum at the same dilution did not. In one experiment, the uterine fluid of an immunized rat inhibited the action of the enzyme. A discussion is pre-

sented of the relationship of these findings to clinical observations on problems of sterility and artificial insemination in humans.

ADDENDUM

Through an oversight a significant reference was omitted. Rowlands (Nature 154: 332, 1944) was able to decrease markedly the number of sperm necessary to successfully fertilize rabbits by artificial insemination when an extract of rabbit sperm containing hyaluronidase was added to the diluted sperm suspensions. This is further evidence that large numbers of mammalian sperm are necessary in the process of fertilization for reasons other than merely insuring the chances for a union of the gametes.

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ULTRAFILTRATION OF URINE FOR COLLECTION AND BIOLOGICAL ASSAY OF EXCRETED HYPOPHYSEAL HORMONES

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THE DEVELOPMENT of a convenient and dependable procedure for bioassay of excreted pituitary hormones has commanded the continued interests of many endocrinologists. The numerous published techniques, and their variations, attest to this fact. Their number indicates, also, that although dependable procedures were evolved some time ago, all of them are laborious enough to stimulate further inquiry. The procedure described here achieves both economy of effort and completeness and accuracy of result, and would, therefore, seem to fulfill a long sought purpose.

Existing methods for concentrating urinary hypophyseal hormones all depend upon precipitation with various reagents (alcohol, tannic acid, tungstic acid, acetone, ammonium sulphate, etc.) and subsequent processing of the precipitate. To insure completeness of recovery and to avoid denaturation of hormone various specialized manipulations (pH adjustment, dialysis, working at cold-room temperatures, etc.) have been employed. The difficulties incident to proper employment of these measures with large volumes of urine apparently have discouraged a good deal of investigation of the urinary pituitary hormones.

That this deterrent has existed for some time seems well demonstrated by the almost complete lack of reliable substantiated information regarding normal rates of excretion of pituitary hormones, other than the gonadotropic factor. Furthermore, interest with but little subsequent investigation has long been expressed in the literature regarding the normal cyclic variation of pituitary hormones during pregnancy, lactation, seasonal and oestrous sexual cycles, climacteric and aging. Pituitary hormones have been implicated in many physiological and morphological pathologies, including cancer, without actual supporting bioassay of any body fluid. The response of the pituitary when different chemical and surgical influences are brought

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to bear upon the organism often have been suggested but most often not measured. In all these instances the usefulness of a properly accurate and convenient assay technique is obvious.

The procedure described here for concentrating urinary hypophyseal hormones has in its favor relatively great simplicity and ease of performance, as well as accuracy and completeness. It is based on the fact that pituitary hormonal proteins comprise part of a relatively minute mass of material of large molecular size in the urine. For this reason physical filtration of the larger molecular entities from the urinary crystalloids should be possible if membranes of proper permeability are available. Such membranes were first prepared from nitrocellulose solutions by Elford (1931). Since Elford's description of a method for preparation of membranes of controlled porosity, to separate particles of different molecular size, this problem has received much study on the part of physical and biological chemists. The procedure for preparation of collodion membranes most closely followed in the present work is that of Wilson and Holiday (1933).

For simpler reference, the procedure for urinary ultrafiltration will be described first in its finally evolved form. This will be followed by a detailed discussion of the data collected during the construction of this procedure, and the factors which have been found to influence it.

METHOD FOR ULTRAFILTRATION OF URINE

A. PREPARATION OF COLLODION MEMBRANES

By dilution of Merck's U.S.P. (4% nitrocellulose) or C.P. (5% nitrocellulose) Collodion a 2% nitrocellulose solution is prepared. The diluent is a mixture of 7 parts by volume absolute alcohol, 3 parts anhydrous ether, and 1 part glacial acetic acid. For slightly more rapid filtration membranes prepared from 1.5% nitrocellulose solutions also may be used though they are mechanically weaker. Only enough diluted nitrocellulose is made to suffice for a week or less, and it must be kept tightly covered.

A perfectly smooth scratch-free disc of good plate glass 6 inches in diameter is floated on mercury in a desiccator over sulfuric acid. Exactly 10.0 cc. of the diluted collodion is placed on the glass disc and allowed to dry 15.0 minutes in the covered desiccator. The collodion-covered disc is then carefully lifted out and slid in a horizontal position into a vessel of water. If the glass plate is properly clean and scratch-free the gelled membrane soon floats free and can be lifted on a piece of filter paper of the same diameter. Membranes may be stored in a vessel of water between pieces of filter paper. Several crystals of thymol must be added to prevent fungus growth.

The glass discs, between occasions of use may be kept in cleaning solution. They are washed off with tapwater, distilled water, drained and dried on edge just before use.

B. THE ULTRAFILTRATION UNIT AND ITS USE

The ultrafilter is a closed steel cylinder of sturdy construction from which the bottom may be unbolted (Fig. 1). It is made by welding a top plate and a bottom collar to a 12-inch length of steam pipe having an inside diameter of 5.0 to 5.5 inches. The bottom plate, which is bolted to the cylinder by six bolts, is recessed to contain a porous glass or metal disc,[2] and is grooved on its internal face to allow flow toward the drainage hole in its center. A gasket to fit between the

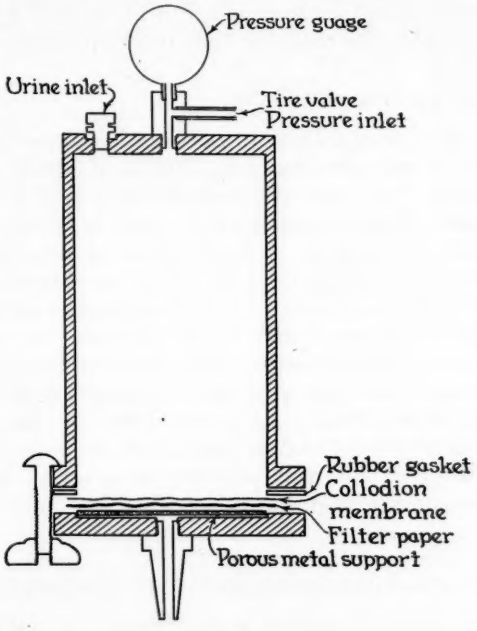

FIG. 1

bottom collar of the cylinder and the bottom plate completes the assembly. The top of the cylinder is tapped to allow entry of urine. It is fitted with a dial pressure gauge into the base of which an automobile tire valve is soldered to allow introduction of pressure. The construction of one of these units presents no difficult problems to any machinist with ordinary equipment.

In practice the collodion membrane, resting on top of a piece of filter paper, is laid upon the porous plate, and the gasket is centered over it. The unit is then bolted together and the measured volume of urine is introduced through the top. Before placing it in the cylinder, the urine is pre-filtered through fine paper, under suction if necessary.

[2] "Oilite," a porous metal made by sintering powdered brass. Manufactured by the Chrysler Corporation, Detroit.

Although it is not essential, the pH may be adjusted just before ultrafiltration with NaOH or HCl until just alkaline to litmus paper, if most rapid filtration is to be achieved. After introduction of the urine the inlet is closed and pressure is added through the tire valve. Most recently we have used a nitrogen tank with a reducing valve as the pressure source. Earlier, a hand tire pump was used with the same results but without the convenience. Pressure of 10 to 15 pounds is introduced at first. The pressure is increased up to a maximum of 100 pounds, as necessary to maintain the steady flow of filtered urine. Collection of the filtrate into a vessel of graduated volume makes it possible to ascertain the rate and time of completion of filtration.

C. PROCESSING OF THE MEMBRANE

Upon completion of filtration, pressure is released and the unit is unbolted. The membrane is carefully lifted and folded and placed into a fifteen cc. centrifuge tube. Alcohol-ether is added immediately and solution of the collodion facilitated by stirring. After centrifugation the supernatant is discarded and the hormone-containing residue is washed four or five additional times with alcohol-ether. After the last washing the residue is dried in low vaccuum. For gonadotropic bioassay, it is extracted twice with small volumes (we use two five cc. extractions) of distilled water or saline solution, and fractions of the combined extracts are administered to groups of four mice, once daily for four days. Ninety-six hours after the first injection the uterus is dissected cleanly and weighed. It is not necessary to dry the residue completely if all traces of ether have been removed. Consequently it is possible to begin the assay of a urine on the same day it is received.

FACTORS WHICH INFLUENCE RATE OF FILTRATION

An entirely practical objective in ultrafiltration of urines is completion of filtration within a reasonable span of time. Aside from inconvenience, extended filtration at room temperatures may involve some loss of hormonal activity due to denaturation.

Obvious factors limiting the rate of passage of fluid are pressure, the area of the membrane, and the dimensions of the interstices or pores through which the fluid must flow. Control over initial porosity of the membrane can be exercised in a variety of ways described in the literature (Elford, 1931; Wilson and Holiday, 1933; Grabar, 1938). In the present work the most convenient method of control was found to be variation of the concentration of the nitrocellulose solutions from which the membrane was made. This matter is dealt with more extensively in a later section of this report.

It may be said in passing that not all marketed collodions have the same physical properties. This factor could not be investigated in an exhaustive manner and the experiments reported here were all done

with Merck's Collodion solutions. Solutions of Dupont's (dry) Parlodion were also found satisfactory. Several other brands of nitrocellulose, both dry and in solution, yielded membranes of such low mechanical strength that they were unsuited for filtration under pressure.

BLOCKING OF COLLODION MEMBRANES

Examination of Figures 4 and 5 reveals a characteristic feature of urinary ultrafiltration—a progressive decrease in rate. It is clear that this decrease in rate of filtration puts a practical limit upon the usefulness of a given membrane. It is also fairly obvious, since this effect is cumulative, that it represents a progressive blocking of available pores by factors contained in the filtering urine. This thesis was demonstrated experimentally and is represented in Figures 2 and 3.

Approximately neutral distilled water was forced through a 2% membrane (made from a 2% nitrocellulose solution) under 15 pounds pressure. A constant rate of passage was found. The water was then replaced in the filter by urine at the same pH and pressure. Twenty cc. of urine were passed through the membrane and then the urine was replaced again by water. A new constant, but slower, rate of filtration of water was found. This is strikingly shown in figure 3 in which the data of Figure 2 are replotted as rates. This demonstrates that the passage of urine through a membrane decreases its permeability to water. The converse experiment also has been done. After initial filtration of urine, twenty cc. of water at the same pH and pressure were passed through a fresh portion of the same membrane as used in the first experiment. This was followed by the urine again. Resumption of urinary ultrafiltration was at the same rate as at the time of introduction of water. This indicates that passage of water alone will not appreciably alter the permeability characteristics of a membrane. It may be pointed out, however, that in Figure 3 it appears that resumed filtration of urine after water is at a slightly higher rate than just before the water filtration. If this be admitted, then it would seem that some of the pore blocking is by substances more soluble in water than in urine—a possible clue to their identity.

A. EFFECT OF HYDROGEN ION CONCENTRATION

Figures 4 and 5 summarize several representative experiments which show the effect of pH on rate of filtration, and, therefore, also on the rate of blocking. Portions of the same urine were adjusted to pH 3.8 and 6.8 (Fig. 4) and placed into separate filters each containing a collodion membrane cut from the same large membrane, and each supplied simultaneously with pressure through a lead from a common pressure reservoir. Since the urine, membrane, and pressure variations are identical in these companion filtrations, the only variable is pH. The neutral urine filters more rapidly than the acid urine, and from Figure 5 it may be concluded that alkaline urine filters even more

rapidly than neutral urine. In fact, it may be calculated roughly that after 60 minutes of filtration about 30% more urine is processed at pH 9.0 than at 3.8. No attempt has been made to elucidate this result,

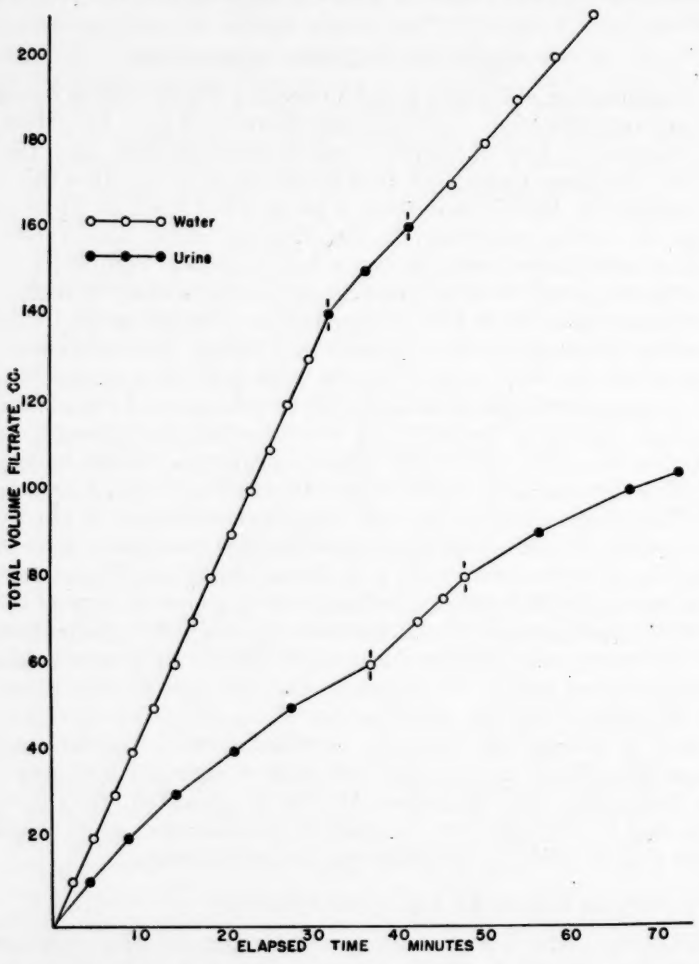

FIG. 2. Effect on accumulation of filtrate of alternate filtration of urine and water through 2% collodion membranes 45 mm. in diameter. Both fluids were adjusted to pH 6.8 before filtration. Pressure of 15 pounds per square inch was used.

but, as a great deal of work by biophysical students of ultrafiltration has indicated, it is likely related to differences in membrane adsorption at different values of pH. Such tests have been repeated on a large number of urines. All have shown accelerated filtration at alkaline pH values, although the degree of response is somewhat variable.

To take advantage of this factor, without the inconvenience of meticulous adjustment of pH, we have adopted the practice of adding acid or base until the urine sample is just on the blue side of the litmus paper reaction. If the urine sample is small even this means of hastening filtration is usually unnecessary.

B. BLOCKING BY PARTICULATE MATTER

It is obvious that settling of gross sediments upon the collodion membrane will decrease its efficiency. Experience has shown that colloidal suspensions, probably even more than particulate matter of larger dimension, will very rapidly decrease the available area of an

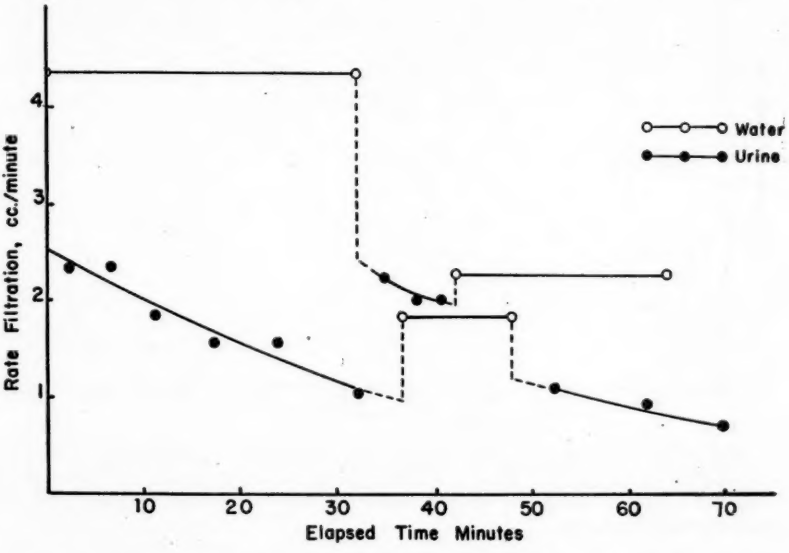

FIG. 3. Effect on rate of filtration of alternate filtration of water and urine through 2% collodion membranes (45 mm. diameter), under 15 pounds pressure. Both fluids were adjusted to pH 6.8 before filtration.

ultrafilter membrane. Numerous observations of a qualitative character have been made on portions of the same urine containing, or freed from, colloidal matter. The difference in loss of rate of filtration is quite large.

For this reason it is rewarding to filter cloudy urines through very fine paper, with vacuum if necessary, to clarify them before introducing them into the ultrafilter.

C. BLOCKING BY DISSOLVED MATTER

A residue of surprising size is obtained after ultrafiltration of a clear twenty-four hour urine sample. In the centrifuge tube, after re-

moval of collodion it usually occupies about one-half cc. The rate of filtration is roughly inversely proportional to the volume of residue obtained. Since this residue appears related to the blocking effect in ultrafiltration, and since it must obviously include some smaller molecular entities, its composition would be interesting to ascertain. Pre-

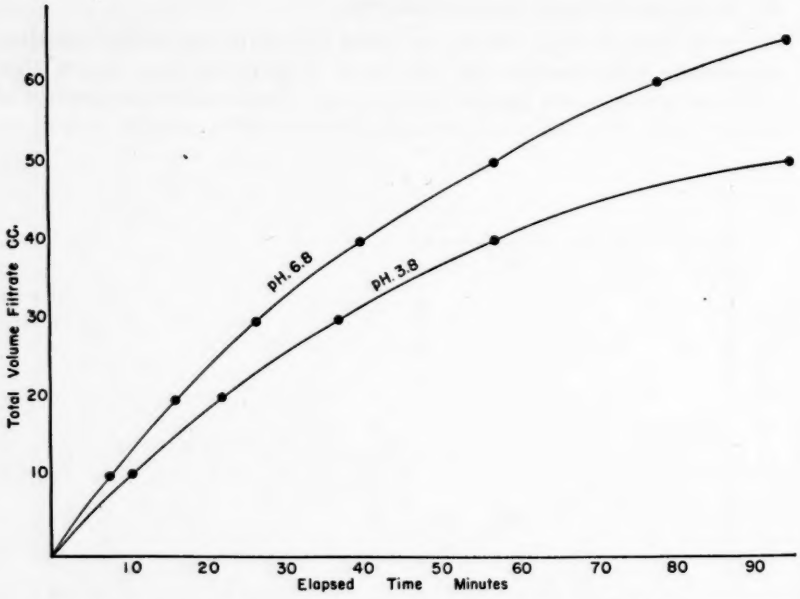

FIG. 4. Effect of pH on rate of filtration. Portions of the same urine specimen were adjusted to pH 3.8 and 6.8. They were filtered simultaneously through portions of the same 2% collodion membrane (45 mm. diameter) under 15 pounds pressure from the same pressure reservoir.

liminary tests indicate that it contains uric acid, but further analysis has not yet been possible.

Urines rich in albumin decrease very rapidly in filtration rate. They give volumes of residue, furthermore, which are out of proportion to their protein content, sometimes as much as 1.5 cc. This behavior indicates that perhaps as filtration becomes more complete, and pores become progressively more clogged, that membrane permeability may be decreased so far below the original value that much smaller molecules and molecular aggregates may be filtered out, contributing to the residue obtained.

The problem of blocking by dissolved matter becomes extreme only in urines with a high protein content. In these cases the problem may be met by separating the urine sample into two, or even three, portions which are filtered through separate membranes.

D. RELATION OF PRESSURE TO BLOCKING

Elford and Ferry (1934) and others have shown that the blocking effect, and consequent decreased permeability, are greater at higher pressures. For this reason, in the ultrafiltration of urine, the initial

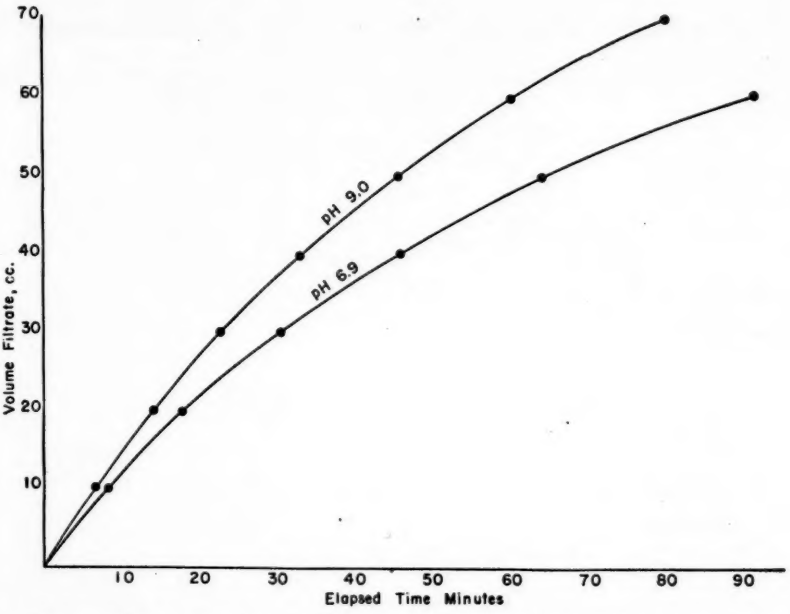

FIG. 5. Effect of pH on rate of filtration. Portions of the same urine specimen were adjusted to pH 6.9 and 9.0. They were filtered simultaneously through portions of the same 2% collodion membrane (45 mm. diameter) under 15 pounds pressure from the same pressure reservoir.

pressure used is low (10 to 15 pounds) and it is increased only as required to maintain the flow of urine through the filter.

RECOVERY OF HORMONAL ACTIVITY IN ULTRAFILTRATION RESIDUES

The usefulness of the ultrafiltration technique as a basis for bioassay of excreted protein hormones can, after all, be gauged only by the completeness of its yield of filtered hormone. Consequently, the evaluation of this feature has been a primary task.

In determining the degree or percentage of hormonal retention or leakage of collodion membranes two approaches have been made. In one series of tests carefully assayed solutions of chorionic or pregnant mare serum gonadotropin[3] were filtered through collodion membranes

[3] Chorionic gonadotropin, Antuitrin-S, was kindly supplied through Dr. Oliver Kamm of Parke, Davis, and Co. Pregnant mare serum gonadotropin, Gonadin, was supplied through the courtesy of Dr. D. H. Wonder of Cutter Laboratories.

of varying porosity. The hormonal activity in the membrane residue and in the filtrate was then assayed for comparison with the original unfiltered solution. In another series of tests, the gonadotropic activity in ultrafilter residues from a large number of twenty-four hour urine samples was determined. These assay values were compared

TABLE I. TESTS FOR HORMONAL RETENTION IN COLLODION ULTRAFILTRATION OF CHORIONIC GONADOTROPIN

Exp. No.	Filtered Hormone	Collodion Membrane	Dose Per Assay Mouse Equivalent[1]			Assay Mouse Uterine Wt. Avg. Mg.	Control Mouse Uterine Wt. Avg. Mg.	Remarks
			Unfiltered Sol.	Filtrate	Residue			
1.	Antuitrin-S #952 0.1 mg. per cc. of normal saline		.005 mg.			13.9	9.5	Loss of hormone through the 2% membrane is<10%. Activity of the residue indicates that loss, if any, is much <10%.
			[2].009 mg.			20.7	9.5	
			.018 mg.			26.2	9.5	
			.040 mg.			47.6	12.4	
			.100 mg.			49.3	12.4	
				.100 mg.		12.6	12.4	
		2%			.100 mg.	48.3	12.4	
		2%			.040 mg.	45.5	12.4	
		2%						
2.	Antuitrin-S #952 prepared as above	1%		.100 mg.		31.0	8.5	Considerable loss of chorionic gonadotropin through 1% membrane. (Percentage not calculable from data.)
		1%			.100 mg.	44.0	8.5	
3.	Antuitrin-S #952 prepared as above	1.3%		.100 mg.		20.5	8.5	10% loss of purified chorionic gonadotropin in saline, through a 1.3% membrane.
		1.3%			.100 mg.	44.0	8.5	
4.	Antuitrin-S #952 0.1 mg. per cc. of boiled urine	1.3%		.100 mg.		11.0	9.5	Urinary solution of chorionic gonadotropin loses <10% activity through a 1.3% collodion membrane. Activity of residue indicates practical completeness of retention.
		1.3%			.100 mg.	51.5	9.5	

[1] The equivalent dosages in these columns are those that would obtain if filtration were complete (filtrate column) or membrane retention were complete (residue column.) Assay values obtained indicate the degree of the deviations from these assumptions and permit calculation of percentage loss in filtration.
[2] This particular standardization has been repeatedly determined and confirmed.

with those reported in the literature by workers using other techniques.

Table I summarizes some pertinent ultrafiltration recovery experiments with chorionic gonadotropin (Antuitrin-S). The solution of hormone contained 0.1 mg. per cc. of normal saline. It was carefully and accurately determined that the equivalent of .009 mg. of hormone in this solution was equal to one "mouse-uterus unit" (i.e., elicited a 100% increase in uterine weight over that of untreated 24-day old mice). After ultrafiltration of the solution through a membrane prepared from 2% nitrocellulose (Merck Collodion) solution there was no detectable hormonal activity in the filtrate. On the other hand, (Table I) when the ultrafiltration was through a 1.0% membrane, considerable (per cent not calculable) chorionic gonadotropin was found in the filtrate. If a 1.3% membrane was used, about 10% of the gonadotropic activity could be found in the filtrate. However, if the

hormone was dissolved in boiled urine instead of saline solution, and filtered through a 1.3% membrane, even this 10% leakage of gonadotropic activity was not detectable in the filtrate. This is not surprising when it is recalled that the passage of urine through a collodion membrane rapidly reduces its effective pore size and permeability. Since hormonal loss through the 1.3% membrane from urine solution is considerably less than 10% we have felt justified in using 1.5% and 1.3% membranes for routine gonadotropic assays of human urines, especially because they permit somewhat more rapid filtration.

TABLE II. TESTS FOR HORMONAL RETENTION IN COLLODION ULTRAFILTRATION OF PREGNANT MARE SERUM GONADOTROPIN

Filtered Hormone	Collodion Membrane	Equivalent Dose Per Assay Mouse[1]			Uterine Wt. Assay Mice Avg., mg.	Uterine Wt. Control Mice Avg., mg.	Remarks
		Orig. Unfil. Solution	Filtrate	Residue			
Gonadin, #3410 B 1860 diluted with saline to rated concentration of 10 "units" per cc.	1.5%	1.0 "unit" [2]0.25 u.	20 u.	1.0 u.	49.0 24.0 10.4 59.0	9.0 9.0 10.0 10.0	Loss of Veterinary Gonadin through a 1.5% membrane is <1.25%. Apparent retention of hormone on membrane is complete.
	1.5%			.5 u.	46.0	10.0	
a.) Gonadin #3410 B 1860 (Same as above)	1.0%		10 u.		42.0	9.0	Considerable activity of PMS passes through a 1.0% collodion membrane. Most of this can be stopped by a 1.33% membrane.
b.) Hormone containing filtrate of 1% membrane filtration	1.33%		10 u.		14.5	9.0	
Gonadin #B2552 (PMS preparation in which separation of hormone from plasma protein is quite complete) diluted with saline to rated concentration of 50 "units" per cc.	1.33%	1.0 u. 0.5 u.	50 u.	1.0 u.	18.5 11.0 9.0 20.0	8.0 8.0 8.0 8.0	Less than 2% loss of PMS gonadotropin through 1.33% collodion membrane. Apparent retention by membrane is complete.

[1] The equivalent dosage in the "filtrate" and "residue" columns are those that would obtain if filtration were complete (Filtrate column) or retention were complete (Residue column). The actual deviation from these assumptions is indicated by the assay value obtained in each case. Comparison of the assumed versus the actual dosage (as revealed by assay) permits calculation of the degree of filtration.

[2] This particular datum has been repeatedly confirmed.

Table II summarizes similar recovery tests with pregnant mare serum gonadotropin (PMS). The preparation used (Gonadin) bears a labelled gonadotropic titer in international units. In repeated determinations 1.0 international unit of Gonadin was found equal to 4.0 mouse-uterus units. Ultrafiltered through a 1.5% membrane this preparation did not appear in detectable amounts in the filtrate, indicating that the loss of PMS under these particular conditions is less than 1.25%. An appreciable quantity of PMS was lost, however, through a 1.0%, but not a 1.3% membrane. One preparation of Gonadin (B2552) in which hormonal separation from plasma proteins is quite complete, showed less than 2% tendency to pass through a 1.3% collodion membrane.

Table III is a compilation of gonadotropic titers in ultrafilter resi-

TABLE III. GONADOTROPIC ASSAYS OF ULTRAFILTRATION RESIDUES OF HUMAN URINES

Subj.	Age	Sex	Clinical Features	Collodion Membrane	Fraction of 24 hr. urine given assay mouse[1]	Uterine Wt. assay mice Avg., mg.	Uterine Wt. Controls Avg., mg.	Remarks Titer in mouse-uterus units
1.	4	Male	Chronic pneumonia	1.3%	140/950	11.0	8.0	Less than 5 u./24 hr.
2.	3	Male	"Renal dwarfism"	1.3%	400/2250	16.4	8.0	5.5 u./24 hr.
3.	18	Male	Normal	1.3%	180/1510	19.5	7.5	ca 8 u./24 hr.
4.	18	Male	Normal	2.0%	132/900	19.2	9.0	7.6 u./24 hr.
					92/1320	17.0	8.0	14.3 u./24 hr.
5.	19	Male	Melanotic tumor		189/1890	17.0	7.0	ca. 10 u./24 hr.
			Preoperative	1.3%	80/1165	15.5	7.0	12.5 u./24 hr.
			Post Preoperative					
6.	28	Male	Normal	2%	200/1150	16.2	9.5	slightly less than 6 u./24 hr.
7.	30	Male	Normal	1.5%	82/1220	20.0	9.5	15 u./24 hr.
				1.5%	330/1640	22.2	9.0	5 u./24 hr.
				1.3%	148/1480	19.1	9.0	10 u./24 hr.
				1.3%	110/1105	17.0	8.0	10 u./24 hr.
				1.0%	118/1180	23.0	9.0	10 u./24 hr.
				1.0%	190/940	21.0	10.5	5 u./24 hr.
8.	33	Male	Normal	2%	400/1400	66.0	9.5	At this dose large follicles and corpora lutea in ovaries of assay mice
					100/1400	23.2	10.0	14 u./24 hr.
9.	37	Male	Normal	1.3%	101/1010	16.	9.0	Slightly less than 10 u./24 hr.
10.	79	Male	Prostatic carcinoma	1.3%	60/1100	21.0	9.0	18.4 u./24 hr.
11.		Male	Normal males pooled urine alcohol concentrate previously assayed at 7.1 u. per liter (#WN32)	2%	140/(—)	25.	9.5	At least 7.1 u. per liter
12.	49	Female	Menopause	1.3%	90/1150	16.5	8.0	12.8 u./24 hr.
				1.3%	101/1010	18.0	8.0	10 u./24 hr.

[1] Only significant dosages are listed to save space. In the fractions in this column the denominator is the actual 24 hour urine volume; the numerator is the equivalent volume of urine given each assay mouse.

dues of 24-hour urines from a series of normal and clinical subjects. In the case of all adult males in this group the titers fall between 5.0 and 15.0 mouse-uterus units per 24 hours. This figure compares quite

favorably with the numerous published titers of normal male urine obtained from various kinds of urinary precipitates.

Experiment 11 in this series is probably a more critical indicator for comparative purposes than the other data. In this case a solution containing a redissolved alcoholic precipitate of pooled normal male urine was ultrafiltered through a 2% membrane. This precipitate had been assayed previously and a gonadotropic activity found equivalent to 7.1 mouse units per liter. The ultrafilter residue was found to contain an equivalent activity of slightly more than 7.0 mouse units per liter, indicating a 100% recovery.

In subject 7 of Table III it is interesting that titers obtained from 1% membrane residues are similar to those from 1.3% and 1.5% membrane residues. This is probably due to the blocking effect upon pore diameter during urinary ultrafiltration. It further justifies the use of 1.3% membranes for routine urinary ultrafiltration even though it is known that under certain circumstances gonadotropic hormones in aqueous solution will penetrate such a membrane to a limited extent.

DISCUSSION

Although the ultrafiltration technique has been applied here only to concentrating urinary gonadotropins, it is quite clear that the concentrates so obtained can, at the same time, be used as a basis for assay of the other large molecular hormones. Some work, still unpublished, has been completed in which ultrafiltration concentrates have been tested for intermedin (chromatophorotropic) activity. Since there is still some question in the literature as to the true identity of urinary substances which affect melanophores these data are being withheld for further study. Furthermore, preliminary results indicating lactogenic hormonal activity have been obtained by A. S. Albrieux (unpublished) with one urinary ultrafiltration residue of normal male urine. He found a measurable amount of lactogenic hormonal activity in an assay using the crop sac of the pigeon.

SUMMARY

A simplified method for concentration and assay of excreted protein hormones has been developed and is described in detail.

Urine or aqueous solutions of gonadotropic hormones are ultrafiltered through a collodion membrane under pressure. The membrane is dissolved in alcohol-ether in a centrifuge tube and the untrafiltered residue is freed from traces of collodion by alternate fresh washes of alcohol-ether and centrifugation. The dried residue is extracted twice with water and the combined extracts are bioassayed.

Normal male urines so processed and assayed for gonadotropin in 24-day old female mice gave values of 5 to 15 mouse uterus units per 24 hours, a unit being the amount of hormone necessary to produce a 100% increase in uterine weight in immature mice. Conditions for

greatest rapidity of ultrafiltration and completeness of hormonal recovery have been worked out and are discussed in detail. In critical tests complete retention by the collodion membrane has been found for known solutions of chorionic gonadotropin, pregnant mare serum gonadotropin, and normal male urinary gonadotropin.

In preliminary experiments applicability of this technique to the study of other excreted hypophyseal hormones is indicated.

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THE EFFECTS OF PANCREATECTOMY ON GLYCOSURIA AND KETOSIS IN DOGS MADE DIABETIC BY ALLOXAN[1]

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FOLLOWING the experiments of Dunn, Sheehan, and McLetchie (1943,a) demonstrating that alloxan was capable of producing necrosis of the islets of Langerhans in rabbits, it became evident that this substance could be used for the production of permanent experimental diabetes in several species (Dunn, Sheehan, and McLetchie; 1943,b; Bailey and Bailey, 1943; Goldner and Gomori, 1943). Of particular interest was the work carried out on dogs (Goldner and Gomori, 1943), since the metabolic state which resulted from alloxan administration was found to be associated with a histological picture of selective necrosis of the beta cells without damage to the other components of the islet or acinar tissue. Moreover the hyperglycemia and hyperlipemia in these dogs was considered by the authors to be even more severe than that usually seen in pancreatectomized animals, and, although the suggestion was made that this difference was the result of the drug's toxic action on the liver, it seems paradoxical that, in the absence of insulin therapy, these dogs lived a rather long time (16–48 days), without ever developing severe ketosis.

The striking specificity of alloxan for the beta cells in dogs offers a technique for investigating the possible endocrine activity of other pancreatic tissue. Such an investigation was instigated by the suggestion of significant metabolic differences between alloxan diabetes and that which follows surgical removal of the pancreas in experimental animals. Consequently, in the experiments to be reported, dogs were made diabetic with alloxan and appropriate metabolic studies were carried out. Some of the animals were then pancreatectomized while others were subjected to pancreatic duct ligation.

MATERIALS AND METHODS

Animals

Healthy mongrel dogs of the "hound" type were housed individually in metabolic cages from which 24-hour urine collections could be made. Each dog's diet was originally adjusted so as to maintain a constant body weight

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and, except for the first few days after operation, was not altered throughout the course of the experiment. In general this allowed per kilogram of body weight 15 grams of ground, cooked horsemeat, 2.5 grams of dried bread crumbs and .5 grams of Brewer's yeast. The ration was given in two daily meals at twelve-hour intervals, water being supplied ad lib.

Insulin Control

Regular insulin was injected twice daily in equal doses immediately after each meal. By allowing the persistence of a slight glycosuria, it was possible to avoid hyper-insulinism and to establish a positive level of control against which the insulin requirement could be redetermined for each type of experimental diabetes. Insulin adequate to maintain a constant glycosuria somewhere within the range of 2 to 12 grams daily was accepted as constituting the insulin requirement.

Surgery

Ligation and division of the pancreatic ducts as well as total pancreatectomy were conducted under ether anesthesia. One pancreatectomy, attempted in an alloxan-diabetic dog which had undergone duct-ligation two weeks previously was found impossible because of adhesions, and this type of experiment was abandoned.

Chemistry and Pathology

Blood sugar was determined by the micro-method of Folin and Malmros (1929) employing the Klett photoelectric colorimeter. Urinary glucose was measured by titration with Benedict's quantitative reagent; and determinations of the combined urinary acetone and acetoacetic acid ("acetone bodies") were carried out according to the technique described by Behre (1940).

Pancreatic tissue obtained from the extirpated glands or from biopsy at the time of the duct ligations was prepared for differential islet-cell staining by Gomori's chrome hematoxylin and phloxine method (1931). Liver fat was demonstrated with the scarlet red frozen section technique. The findings in the islet tissue, namely the disappearance or degeneration of the beta cells with survival of the alpha cells were the same as those described by other investigators (Goldner and Gomori, 1943, 1944), and will not be discussed further.

Alloxan and Alloxan Diabetes

A number of different samples of alloxan monohydrate were used in these studies, including 12 from Eastman Kodak Co., 2 from the American Cyanamid Co. and one from the Pfanstiehl Chemical Co. In dosages ranging from 55 to 75 mgm. per kilogram, the compound, having been freshly made up into 5% aqueous solution, was rapidly injected into a leg vein.

The daily glucose excretion was taken as the index of severity of the resulting diabetes, and it was thought that any dog excreting less than 5 grams of glucose per kilogram of body weight per day was not suitable for this investigation of presumably complete insulin deficiency. There is, of course, no assurance that even the selected dogs did not retain some insulin-producing pancreatic tissue. Of 43 dogs injected, only 7 developed a high degree of

glycosuria without serious complications. Twenty-three dogs excreted a lesser quantity of glucose and were discarded, while the remainder developed toxic renal damage and uremia. Whether the failure to reproduce the state of severe uncomplicated diabetes consistently was a manifestation of individual animal variation or was due to differences in the samples of alloxan is not clear.

RESULTS

The following are the protocols of the seven dogs which were studied in detail.

Alloxan Diabetes (Fig. 1)

Dog #1 was an 11 kg. male that received 55 mgm. per kilo of al-

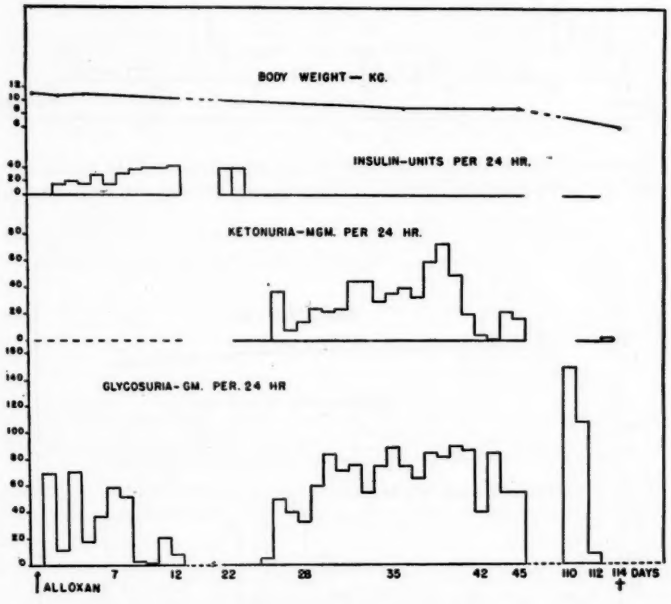

FIG. 1. The course of alloxan diabetes.

loxan. This dog lived 114 days from the date of alloxan treatment; the last 91 days were without insulin therapy. Throughout the experiment the animal maintained an excellent appetite, but gradually lost 5 kg., becoming dehydrated, emaciated, and weak. Forty units of insulin controlled an average glycosuria of 67 grams per day, and after the withdrawal of insulin, the ketone body excretion averaged 36 mgm. in 24 hours.

Alloxan Diabetes Followed by Ligation and Division of Pancreatic Ducts (Fig. 2 and 3)

Dog #2 was a 10 kg. female which was subjected to pancreatic duct

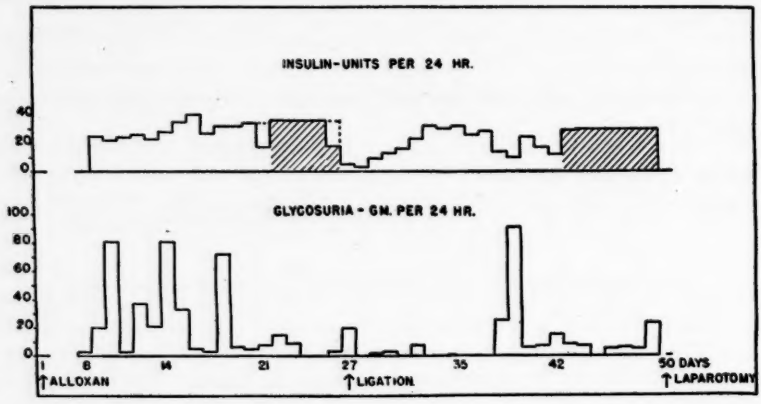

FIG. 2. The course of alloxan diabetes followed by interruption of the pancreatic ducts. Cross-hatched areas indicate region in which insulin control has been established.

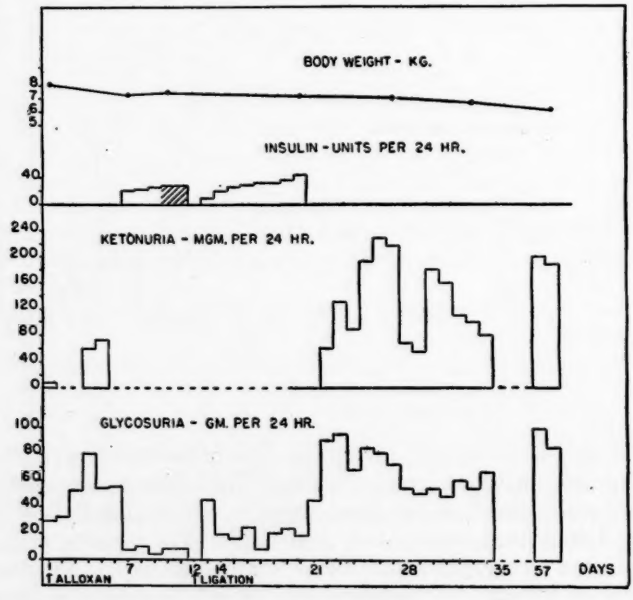

FIG. 3. The course of alloxan diabetes followed by interruption of the pancreatic ducts. Cross-hatched areas indicate region where insulin control has been established.

ligation 27 days after receiving 50 mgm. per kg. of alloxan. This was the first animal to be studied and was the one selected to undergo pancreatectomy after recovering from the ligation. Only the insulin requirement was followed and this dropped from 35 units in the alloxan state to 30 after the ligation.

Dog #3, an 8 kg. male, was subjected to duct ligation 12 days after receiving alloxan. Prior to operation and before insulin was administered, there was glycosuria averaging 50 grams in 24 hours with a

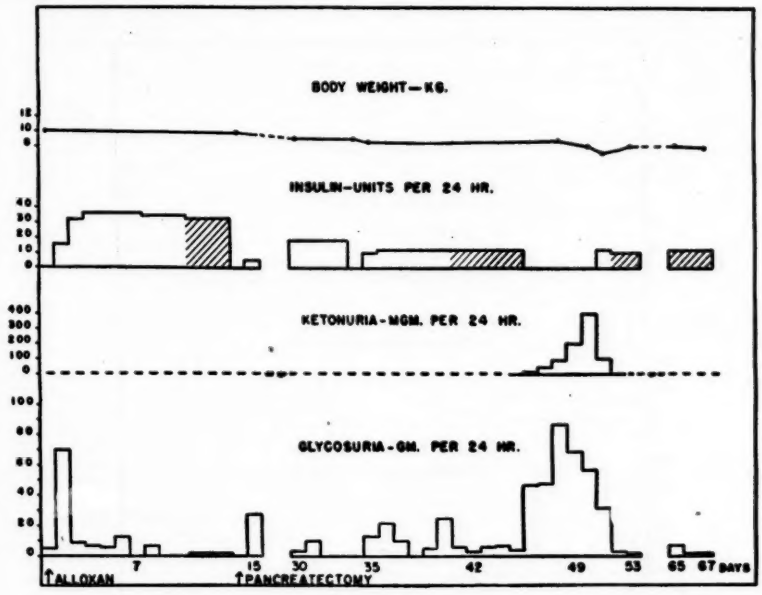

FIG. 4. The course of alloxan diabetes followed by total pancreatectomy. Cross-hatched areas indicate region where insulin control has been established.

daily ketonuria of 7 mgm. Twenty-eight units of insulin were adequate. Following ligation, 44 units were insufficient to control the glycosuria, which, upon the withdrawal of insulin, rose to 83 grams per 24 hours. Although the ketone body excretion rose somewhat to 200 mgm. a day, this average has been maintained without any suggestion of concomitant acidosis. At present, 90 days after the cessation of insulin treatment, the animal is active and healthy, though there has been a gradual loss of 3 kg. in body weight.

Alloxan Diabetes Followed by Pancreatectomy (Fig. 4, 5, 6)

Dog #4 was depancreatized 14 days after being made diabetic with alloxan. This animal was immediately started on insulin therapy without any initial study of glycosuria and ketonuria in the untreated

alloxan phase. The insulin requirement, which had been 34 units, dropped to 12 units per day following pancreatectomy. When insulin was withheld for 5 days, a mounting ketonuria developed until the dog became comatose on the 5th day with an output of 400 mgm. of acetone bodies. During this period there was an average glucose output of 62 gm. and a marked loss of weight, which was regained when insulin treatment was again instituted.

Dog #5, a 22 kg. male, after receiving 65 mgm. per kg. of alloxan, excreted daily an average of 157 grams of glucose with only 7 mgm.

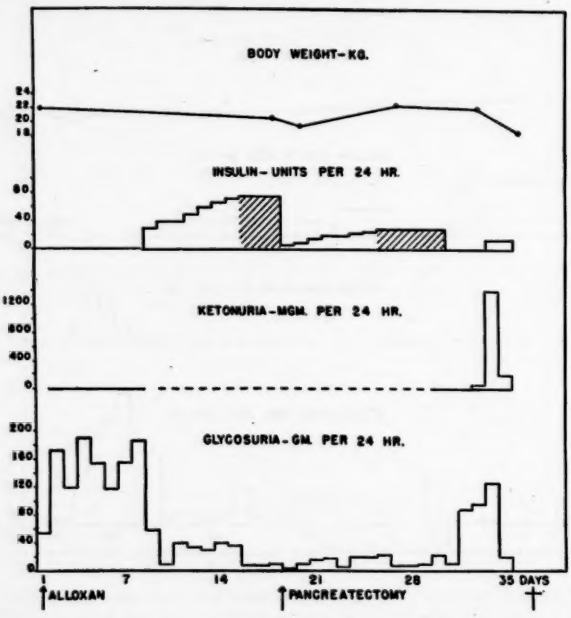

FIG. 5. The course of alloxan diabetes followed by total pancreatectomy. Cross-hatched areas indicate regions in which insulin control has been established.

of ketone bodies. Insulin control was achieved with 76 units daily. Pancreatectomy was carried out on the 18th day, following which the insulin requirement was redetermined as 30 units. When insulin was then withheld, only 53 grams of glucose were excreted per day, though the ketone body output rose rapidly to 1.4 grams on the 5th day without insulin, at which time the animal became comatose. The body weight was the same in the two periods during which the insulin requirement was being measured.

Dog #6, an 18 kg. male, was depancreatized 22 days after being made diabetic with 60 mgm. per kg. of alloxan, and before operation excreted a daily average of 128 grams of glucose. The ketone output rose higher than in any of the other alloxan-diabetic dogs studied, reaching 490 mgm. on the 9th day after alloxan. This ketonuria then

rapidly subsided until the 16th day when only 34 mgm. were lost. The insulin requirement measured preoperatively was 60 units. After operation only 20 units of insulin were necessary, the daily glycosuria was 53 grams, and ketonuria increased steadily until the 7th day after withdrawal of insulin, when, with a ketone output of 1.4 grams, the animal died following the development of acidosis and coma.

Dog #7 was a 9.5 kg. male treated with 55 mgm. per kg. of alloxan, and pancreatectomized 31 days later. The original insulin requirement

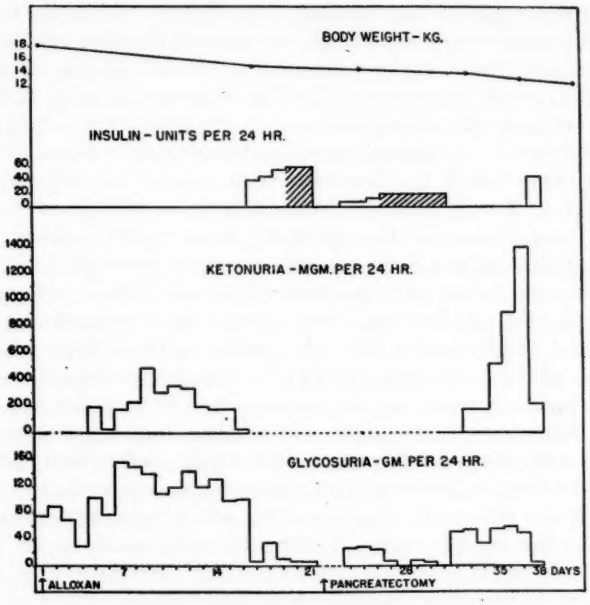

FIG. 6. The course of alloxan diabetes followed by total pancreatectomy. Cross-hatched areas indicate region in which insulin control has been established.

of 36 units was reduced to 12 units after operation. Ketonuria preoperatively ranged from 0 to 20 mgm. When insulin was withheld after operation, the animal died in 5 days, attaining a maximum ketone body excretion of 710 mgm.

DISCUSSION

From these data it is apparent that the diabetes of alloxan-treated dogs is characterized by very severe glycosuria as well as by an extraordinarily high insulin requirement. In terms of these criteria, alloxan diabetes is considerably more severe than that which follows total pancreatectomy in the same species. Alloxan-treated dogs, on the other hand, live a great deal longer than do depancreatized ones in the absence of insulin treatment. They maintain excellent health for long periods and fail to go into diabetic coma. Their survival ap-

pears to be related to a very low urinary ketone body excretion. Pancreatectomy in these animals results in a diminution of the daily glycosuria and a reduction of the insulin requirement to about one-third of its preoperative value, even when body weight and dietary intake remain constant. If insulin is withheld after removal of the pancreas, the dogs rapidly develop progressive ketosis and within a few days pass into the characteristic coma of depancreatized dogs. Ligation of the pancreatic ducts, unlike pancreatectomy, has little or no effect on the course of alloxan diabetes.

In considering the mechanism behind these findings, certain observations which have been made in connection with another type of experimental diabetes are pertinent. In 1937 Young showed that permanent, severe diabetes could be produced in dogs by the repeated injection of large doses of pituitary extract. This state, which persisted indefinitely after the injections were discontinued, seems remarkably similar to that which has been observed in dogs following alloxan administration. Young (1939) summarized these findings as follows:

"The metabolism of these pituitary diabetic dogs differs from that of the depancreatized dogs in some respects, most strikingly in that pituitary animals are able to survive for long periods without insulin therapy, despite the fact that they apparently require more insulin for the control of glycosuria than do depancreatized dogs which under similar conditions may quickly die when insulin treatment is stopped."

It is significant that the histological studies on pancreases of pituitary-diabetic dogs reveal, just as in alloxan diabetes, degeneration of the beta cells without damage to the alpha cells (Ham and Haist, 1941). The beta cells have been generally accepted as the source of insulin in the pancreas. Consequently, since beta cell damage is the only constant morphological finding in both these types of experimental diabetes, it is reasonable to assume that the syndrome represents a simple, uncomplicated insulin deficiency.

In order to offer a satisfactory explanation for the difference between the physiological picture of pure beta cell deficiency and that which characterizes surgical ablation of the pancreas, it seems necessary to postulate the existence of a second pancreatic hormone which possesses at least two types of activity: a hormone which, in the first place acts, in opposition to insulin, to increase the blood sugar and promote glycosuria and, secondly, prevents ketosis and coma in the insulin-deficient animal. The latter effect must be due in some way to the inhibition of ketogenesis, for there is ample evidence that even in pancreatectomized animals, the utilization of ketone bodies proceeds normally (Stadie, 1941). To what extent these two types of activity are related is impossible to decide on the basis of the present evidence. The fact that neither the interruption of the pancreatic ducts nor the administration of alloxan abolishes these effects suggests that the facor is elaborated by a pancreatic endocrine tissue, the most likely being the alpha cells.

The alpha cells of mammals are morphologically distinct struc-

tures possessing granules which are definitely suggestive of secretory activity. The morphological characteristics as well as the solubility and staining reactions of the granules are presumptive evidence of a chemical function differing from that of the beta cells (Bensley, 1911). The only suggestion which has been made regarding the possibility of an alpha cell secretion comes from the work of Dragstedt and his co-workers on *Lipocaic*, which is believed to be necessary for the prevention of fatty metamorphosis of the liver and maintenance of life in pancreatectomized, insulin-treated dogs (Dragstedt, van Prohaska and Harms, 1936). This factor may be supplied either by orally-administered raw pancreas or in the form of a fat-free alcoholic extract of the gland. The preparation is considered to be an extract of the alpha cells. Studies have not been made to determine whether the effects to which attention has been directed in the present paper are due to lipocaic. Whereas Dragstedt and his co-workers found that their preparation would neutralize the effects of ketogenic anterior pituitary extracts (Julian, van Prohaska, Vermeulen and Dragstedt, 1943), it did not influence the ketosis and duration of life of pancreatectomized dogs receiving no insulin (Dragstedt *et al.*, 1941).

The finding that the insulin requirement of the alloxan-diabetic dog is much greater than that of the depancreatized animal would seem to indicate that the amount of exogenous insulin necessary to prevent glycosuria in depancreatized animals does not represent the actual output of insulin by the pancreas under normal circumstances. Rather the insulin requirement of the alloxan-treated animal would be a more accurate measure of this quantity. Consequently it may be fallacious to draw conclusions on the basis of changes that occur in the depancreatized dog under so-called insulin control. The fatty liver, for example, which develops in this situation may be due to a relative insulin deficiency despite the fact that the blood sugar is apparently controlled. The finding that extracts of the pancreas prevent such changes does not rule out this possibility since other lipotropic factors have a similar beneficial effect.

The concept that two hormones are secreted by the pancreas may possibly have important clinical implications, and it is conceivable that such a mechanism may be related to the striking variability which characterizes human diabetes. It has been shown, for example, that the insulin requirement of many diabetics is much higher than that which results from total pancreatectomy in the human (Goldner and Clark, 1944). It is well known, moreover, that while some diabetics develop ketosis readily when insulin is withheld, others do not. The question arises of whether ketosis exists in naturally-occurring diabetes as a result of insulin deficiency alone or whether the absence of a second factor is necessary for this to occur. If the second factor is supplied by the alpha cells, pathological evidence would support the latter theory, for, in those diabetics who show histological pancreatic change, there is hyalinization involving all cells of the islet.

Another suggestion that these mechanisms may be operating in

the human derives from certain cases of diabetes such as that reported by Cushing and Davidoff in 1927. The patient was an acromegalic in whom hyperpituitarism had been arrested by the removal of an eosinophilic pituitary adenoma. This man exhibited very severe hyperglycemia and glycosuria, excreting over 300 grams of glucose per day, but never showed urinary ketone bodies, and was able to maintain good health without the benefit of insulin, a situation which the authors believed to be common in diabetes with this background. Such cases exhibit a syndrome identical in etiology and clinical picture to that described by Young in dogs, and consequently are representative of the condition which has been defined above as "pure insulin deficiency." Presumably this is caused by dysfunction of the beta cells alone, though proof would only lie in appropriate pathological studies including differential stains of pancreatic tissue.

SUMMARY

Although the glycosuria is more severe and the insulin requirement higher in alloxan-treated than in pancreatectomized dogs, the former are able to live much longer without insulin treatment and fail to develop ketosis and diabetic coma. This has been confirmed by pancreatectomy in alloxan-diabetic dogs following which the insulin requirement is less, but the animals pass quickly into coma when not controlled with insulin. The hypothesis has been proposed that there is secreted by the pancreas a second endocrine factor which acts to increase the blood sugar but to prevent ketosis in the insulin deficient animal. This hypothesis is a tentative one which seems to be the simplest explanation of the data, though it can only be confirmed by the actual preparation of an extract possessing these types of activity.

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EFFECTS OF ALLOXAN IN BIRDS

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INDIANAPOLIS 6

SINCE the discovery by Dunn and his associates (1943) that alloxan produces a selective necrosis of pancreatic islet cells, this remarkable compound has been intensively studied. The literature to the present time has been thoroughly reviewed by Joslin (1945) and Chen (1945). Aside from the reports by Goldner and Gomori (1945) on pigeons, and Garcia Ramos (1944) on turtles, studies of alloxan action have been conducted only in mammals. This is explainable partly on the basis that experimental diabetes mellitus can be produced readily by pancreatectomy in mammals, but results of this operation in birds have been uncertain or conflicting. The work of Koppanyi and co-workers (1926) in fowls, and Mirsky and associates (1941) in ducks attests to the temporary and benign effect of pancreatectomy on carbohydrate metabolism in these species. Recently, however, Nelson, Elgart, and Mirsky (1942) found that depancreatized owls present a fairly typical picture of diabetes. As a whole, birds are less susceptible to insulin than mammals (Chen, Anderson, and Maze, in press).

Surgical removal of the pancreas suffers the disadvantage that the animal is also deprived of pancreatic juice. Consequently, digestive disorders which resist treatment may result in some species. The impaired digestion can even cause death although the carbohydrate metabolism is well controlled. The value of alloxan as a scientific tool is largely the result of its specific action on the pancreatic islets without damage to the acinar tissue. In view of this superiority, we decided to make a study of the effect of alloxan on carbohydrate metabolism of several species of birds, namely, owls, pigeons, ducks, and chickens.

METHODS

Using the procedure of Miller and Van Slyke (1936), blood sugar determinations were made before and at various intervals following intravenous injections of freshly-prepared alloxan solutions. Blood samples were drawn from either the leg veins or wing veins. To determine the effect on carbohydrate metabolism, the birds were given a sub-lethal dose of alloxan. After an interval of several days, if diabetes had not developed, another injection was made. As it became apparent that a given dose or injection

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interval produced little or no effect, the dosage was increased and injections were made more frequently, in some cases daily. A total of 3 horned owls (*Bubo virginianus*), 4 barn owls (*Tyto alba pratincola*), 9 white Pekin ducks, 18 roosters, and 35 pigeons was used in this study.

The median lethal dose of alloxan by intravenous injection was also determined for each species except owls and ducks. Postmortem examinations were made on all animals as soon after death as possible. In case an animal appeared weak toward the end of a day, it was sacrificed for immediate necropsy.

All birds were given usual stock diets and no attempt was made to starve the animals before blood sugar determinations. Owls were fed live mice and were allowed to become accustomed to their cages for about 2 weeks before starting the tests.

RESULTS

Horned owls. Starting with a dose of 100 mg. per kg. of alloxan in 2 birds and 150 mg. per kg. in the third, a rise in blood sugar of 10 to 30 mg. per 100 cc. occurred in 24 hours. The dose was repeated in 48 hours, but the blood sugar of 2 owls fell, and in the other bird a further rise of 15 mg. per 100 cc. was found. Little or no change resulted thereafter, so on the eighth day the dose was increased to 150 mg. per kg. for the 2 owls which had received the smaller dose previously. The third owl was given 200 mg. per kg. of alloxan, but died a few hours later, as did one of the others also. The remaining owl was given 3 more injections in the next 16 days of 150, 200, and 250 mg. per kg. The last dose proved fatal in 1 hour. Aside from a sharp, but temporary elevation on the tenth day, blood sugar levels were normal. Microscopic examination of these animals revealed normal pancreatic islet cells. No lesions were noted grossly in other organs.

Barn owls. Doses of 50 to 150 mg. per kg. of alloxan were given to 4 birds at the start. Thereafter the dosage was increased as high as 200 mg. per kg. The owls received from 2 to 4 injections each and lived from 3 to 23 days following the first dose. Little change occurred in the blood sugar of 3 animals. The fourth bird showed an elevation of blood sugar of 14 to 28 mg. per 100 cc. following each alloxan injection, but after this preliminary rise, the blood sugar each time returned to the original level.

In the owl which lived 23 days, slight hydrops of the pancreatic islands and necrosis of the renal convoluted tubules were noted. In addition, the peritoneal surface was dotted with a white powdery-looking material, a condition which appeared similar to that of alloxan-treated pigeons reported by Goldner and Gomori (1945). The kidneys were infiltrated with an apparently similar substance. The owl which died after only 3 days showed the same picture except that the islands of Langerhans were normal and focal necrosis of the liver was present. Post-mortem changes prevented satisfactory pathologic examination of the remaining 2 birds.

Concerning the normal blood sugar of these species, the 4 barn owls averaged 130 mg. per 100 cc. with the range varying from 100 to 146. In 6 horned owls, normal blood sugar varied from 155 to 226, averaging 194. Nelson and associates (1942) reported the normal blood sugar of the horned owl to be 200 to 350. We cannot explain this variation, unless it resulted from a difference in methods of blood sugar determination. Our birds received the same diet as theirs, namely, mice.

Pigeons. Various dosages and injection intervals were tried in pigeons in an attempt to produce permanent diabetes mellitus. An

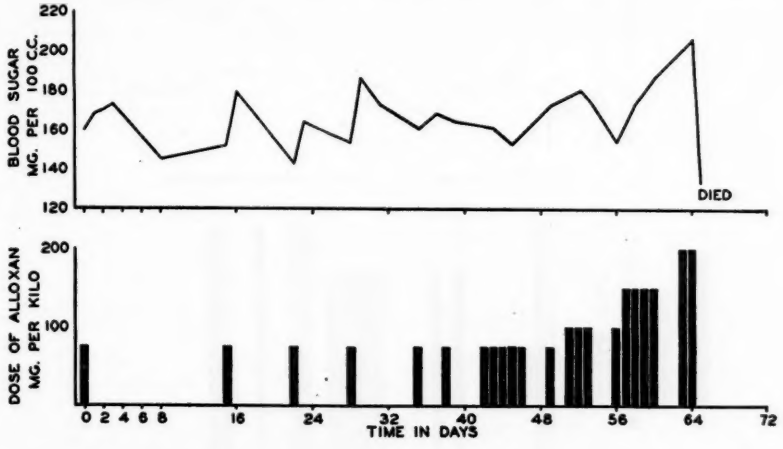

FIG. 1. Effect of repeated intravenous injections of alloxan on blood sugar of a pigeon. Each solid bar represents a dose of the compound.

initial dose of 100 mg. per kg. of alloxan caused death in 30 hours in some instances, with an elevation of blood sugar to approximately 300 mg. per 100 cc. When given 75 mg. per kg. of alloxan, the pigeons did not die, but showed a rise of blood sugar of only 10 to 40 mg. per 100 cc. lasting a day or so. In spite of repeated injections of this and larger doses of alloxan, diabetes mellitus did not develop. Figure 1 shows the record of a pigeon treated with alloxan over a period of 65 days. Other pigeons lived from 25 to 74 days under this regimen. It appears that tolerance to alloxan may have developed in pigeons, since they became able to withstand doses that killed previously untreated animals in a few minutes.

Post-mortem examination revealed essentially the same findings in the pancreas reported by Goldner and Gomori (1945). Some pigeons showed entirely normal pancreatic islets, while in others, hydropic degeneration was found in occasional beta cells. Of the birds which were treated with alloxan over a period of 25 to 74 days, only 1 showed renal tubular necrosis and none revealed deposition of

crystalline material on serosal surfaces. This is in contrast to the findings of Goldner and Gomori. However, in animals which received only 1 injection of 100 mg. per kg. or more and which died in 1 to 2 days, the typical serosal and renal infiltration with crystalline material was present. Apparently, as tolerance to alloxan developed, the pigeon was better able to metabolize or excrete this crystalline substance.

Ducks. In spite of repeated injections of alloxan into 6 birds, the animals survived a period of 19 to 40 days. Starting with doses of

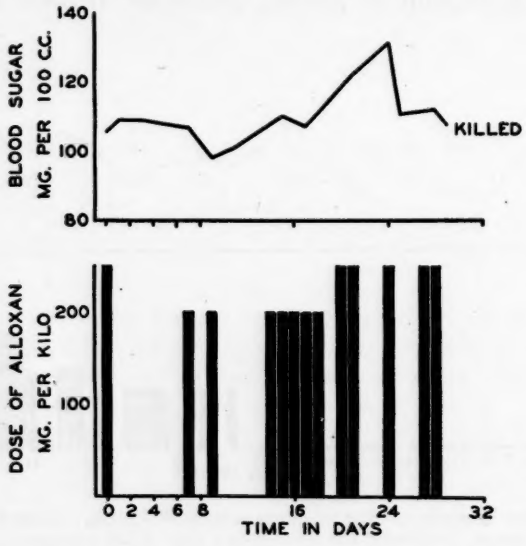

FIG. 2. Action of alloxan in a duck. In spite of repeated intravenous injections of the drug, no definite change resulted in blood sugar.

200 mg. per kg. and gradually increasing to as much as 300 mg. per kg., diabetes did not develop in this species. In fact, changes in blood sugar could be considered to be within the normal range for ducks. A typical record is shown in Figure 2. Like pigeons, these birds appeared to develop tolerance to alloxan. The 3 remaining ducks died in 1 to 2 days from single injections of 250 and 300 mg. per kg. No change in blood sugar was noted in these animals.

In autopsies on 6 ducks, hydrops of a few of the beta cells of the pancreatic islets was found in 5 birds. In no animal was crystalline material seen either on serosal surfaces or deposited in the kidney. Pathologic findings thus were minimal in this species.

Chickens. Single doses of alloxan ranging from 75 to 400 mg. per kg. were given to 18 roosters. No change in blood sugar outside of normal variation was noted. With doses of 300 and 400 mg. per kg., death occurred usually in an hour or less. In other fowls, the blood sugar was

determined several times the first day, and then irregularly for as long as 22 days. At no time was a diabetic tendency observed in any of these roosters.

Microscopic study of the pancreas revealed normal pancreatic islands in the 6 roosters examined. In addition, deposits of white granular material were noted grossly on serosal surfaces and in the kidneys of the 3 roosters which died 2 days after injection. In 2 birds in which death resulted 1 hour after giving alloxan, this condition was not present. Although the white substance was not seen grossly

TABLE 1. THE MEDIAN LETHAL DOSES OF ALLOXAN BY INTRAVENOUS INJECTION INTO PIGEONS AND ROOSTERS

Bird	Dose	Number Died / Number Used	$LD_{50} \pm S.E.$
	mg. per kg.		mg. per kg.
Rooster	75	0/2	
	100	0/2	
	200	3/6	
	250	4/5	199.8 ± 19.4
	300	5/5	
	400	2/2	
Pigeon	50	0/5	
	75	1/8	
	100	3/8	
	125	3/5	111.8 ± 11.8
	150	5/5	
	200	4/4	

in a rooster dying 4 days after the drug, the material was found in the kidney microscopically. Proper dosage and a time factor appeared to be necessary for development of these white deposits. In our birds, doses of 250 to 300 mg. per kg. caused deposition of the substance. Larger or smaller doses were not effective.

Acute toxicity. The median lethal doses ± standard errors of alloxan in pigeons and roosters were determined for intravenous injection. Calculations were made by the method of Bliss (1938), results being shown in Table 1. Because sufficient numbers of owls and ducks were not obtainable, the median lethal doses could not be measured in these species. In ducks, no deaths occurred in 2 birds each on intravenous doses of 100 and 200 mg. per kg., while 1 of 3 animals died from a 250 mg. per kg. dose, and 300 mg. per kg. killed both of 2 ducks. Barn owls survived doses of 50 to 150 mg. per kg., no higher single dose being tested. Likewise no fatalities resulted in horned owls given doses of 100 to 150 mg. per kg. It is obvious, however, that considerable variation in toxicity of alloxan is present in these 5 species of birds. Pigeons and owls appear to be much less

resistant to the drug than roosters and ducks. It is noteworthy also that in our attempts to produce diabetes, doses both above and below the median lethal doses were unsuccessful in producing the disease in these birds.

DISCUSSION

Thus, it appears that aside from occasional temporary elevations of blood sugar, no change was noted in barn owls or horned owls. The rises appeared to be within the normal range of blood sugar for these 2 species. Doses of alloxan which were fatal to pigeons did cause increases of blood sugar, sometimes to 300 mg. per 100 cc. of blood. Smaller doses injected repeatedly for as long as 74 days did not produce diabetes, although elevations of blood sugar within the normal range were noted. Ducks and roosters were very resistant to the action of alloxan, no abnormal changes in blood sugar being produced regardless of dosage. It is clear that alloxan disturbs the carbohydrate metabolism of birds far less than that of mammals.

Post-mortem examination of the pancreatic islet cells revealed minimal changes or no abnormality. Horned owls showed entirely normal pancreatic cells while slight hydrops of the islets was noted in 1 barn owl. In pigeons, entirely normal islet tissue was found in some cases, while others had hydropic degeneration of occasional beta cells. Slight hydrops was seen in these cells in 5 of 6 ducks examined, while no change was found in the islands of roosters. These pathologic changes would appear to be too slight to cause a demonstrable effect on carbohydrate metabolism. The islet tissue of these birds quite obviously is much less affected by alloxan, if at all, than that of mammals.

Confirming Goldner and Gomori, a white crystalline substance was seen on serosal surfaces and in the kidney of pigeons dying from alloxan. This substance was reported by these investigators to be sodium urate, but we made no attempt to determine its chemical nature. A similar condition was also noted in barn owls and roosters but not in horned owls or ducks. The condition developed only when the dose was sufficient to cause death 1 to 2 days after injection. The exact cause of death in the various species was not determined. It seems probable that renal failure was responsible, at least in those birds with crystals deposited in the kidneys. Goldner and Gomori reported an increase of 100 times of the blood uric acid in their alloxan-treated pigeons.

As this paper was going to press, Mirsky (1945) reported on the action of alloxan in ducks. He found that this species did not develop diabetes mellitus, even when the pancreatic islets showed extensive necrosis from alloxan. Our results agree with the observation that no hyperglycemia occurred, but we were unable to demonstrate any necrosis of islet tissue whatsoever.

SUMMARY

Barn owls, horned owls, pigeons, ducks, and chickens were injected intravenously with alloxan in various doses, and usually at several intervals. No changes in blood sugar resulted that could not be considered normal variations except in pigeons which died 1 to 2 days after a single injection. Microscopic examination of pancreatic islets revealed no pathology or minimal changes of the beta cells. In some instances, deposition of a crystalline material was noted on serosal surfaces and in the kidneys. This finding may have accounted for death in those animals in which it occurred. No pathologic alterations were found in other birds which died from alloxan injections.

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THE EFFECT OF ALCOHOL AND POTASSIUM HYDROXIDE CONCENTRATION ON THE REACTION OF 17-KETOSTEROIDS WITH M-DINITROBENZENE[1]

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URINARY EXTRACTS containing a mixture of 17-ketosteroids are assayed colorimetrically by the Zimmermann reaction using a single 17-ketosteroid as a reference standard. If the standard and the other urinary 17-ketosteroids produce significantly different color intensities, per unit weight, such assays will be subject to error. Hence it is important to ascertain whether appreciable differences in the chromogenic values of the various urinary 17-ketosteroids exist, and if these differences can be minimized.

The authors have shown that the relative chromogenic values of several 17-ketosteroids depend on the alcohol and the potassium hydroxide concentration as well as the time used in the Zimmermann reaction. Essentially equivalent colors were produced under some conditions whereas large discrepancies occurred under others (Nathanson and Wilson, 1943). This work has now been expanded to include a larger number of 17-ketosteroids. Observations have been made with a series of reaction mixtures containing selected concentrations of alcohol and potassium hydroxide. Chromogenic values of the individual 17-ketosteroids as well the relation these bear to each other could thus be followed as functions of the alcohol and of the potassium hydroxide concentration.

EXPERIMENTAL

Reaction Mixtures: A total volume of 0.6 cc. was used, consisting of 0.2 cc. of an alcoholic 17-ketosteroid solution, 0.2 cc. of alcoholic 2% m-dinitrobenzene, and 0.2 cc. of aqueous or alcoholic potassium hydroxide. At the appropriate time the reaction mixtures were diluted with 10 cc. of 60% alcohol, and readings made.

Conditions of Experiments: The temperature of the reaction was 25° ±0.2°. Readings were made with an Evelyn photoelectric colorimeter using a Rubicon No. 520 filter. Reagents were as previously described (1). Concentrations of potassium hydroxide and alcohol were varied. Alcohol percentages given are final values, while strength of alkali will be referred to by the normality of the solution added, or 3 times the final normality.

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[1] This is number 604 of the Cancer Commission of Harvard University.

a) Variation of alkali with alcohol constant at 63%: When 95% alcohol was used as the solvent for ketosteroids and m-dinitrobenzene, and aqueous alkali was added, the final alcohol concentration was 63%. Observations were then made when 2.5 N, 5.0 N, 7.5 N, and 10.0 N alkali were added.

b) Variation of alcohol with alkali constant at 2.5 N: 63% alcohol, obtained as above, was the lowest concentration studied, since m-dinitrobenzene precipitated if more water was present. Final concentrations of 80%, 95%, and 100% were also observed.

c) Variation of alkali, with alcohol constant at 100%: All constituents were in absolute alcohol solution. Concentrations of alkali added were 1.0 N, 2.5 N, and 4.2 N. The latter normality was the highest obtainable in absolute alcohol.

Whenever possible, simultaneous observations were made on all the 17-ketosteroids studied in order to show the relationships among them with the least experimental variation. Determinations were made in triplicate or quadruplicate using one appropriate weight of all the 17-ketosteroids. Adherence to Beer's law has previously been demonstrated under reaction conditions at either extreme of our series. All determinations were repeated one or more times in order to substantiate the values obtained.

Substances Observed: Crystalline preparations of androsterone, dehydroisoandrosterone, 3-α-hydroxyetiocholanone-17, isoandrosterone, androstene-3:17-dione, androstenone-17 and chloroandrostenone-17 were studied.[2,3]

Terminology: Chromogenic values are expressed in terms of an absorption constant, K, which is $\frac{\text{2-log galvanometer}}{\text{mg. 17-ketosteroid reacting}}$. Comparison between results obtained under different conditions is thus possible since the same total volume was always used. The term "chromogenic capacity" denotes the absorption obtained with a single 17-ketosteroid at the time of maximum color with any given reagent concentration.

Time of Reading: With each set of conditions the time course of color development was followed by making observations at four or five different time intervals, and the time required to attain maximum color was established. Relationships between chromogenic values of the various 17-ketosteroids have thus been determined both at maximum color and at earlier times. Unless otherwise noted, K values refer to maximum readings with the 520 filter, i.e. chromogenic capacities.

RESULTS

Maximum K Relations: Smooth curves relating maximum K values and potassium hydroxide or alcohol concentration were obtained

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[2] We are indebted to Dr. E. Oppenheimer of Ciba Pharmaceutical Products Inc., Summit, New Jersey, for samples of androsterone, dehydroisoandrosterone and isoandrosterone; to Dr. E. Schwenk of the Schering Corporation, Bloomfield, New Jersey for androstenedione; to Dr. S. Lieberman of the Department of Organic Chemistry, Harvard University, for the etiocholanone, and to Dr. Gregory Pincus of Clark University, Worcester, Mass., for the androstenone-17 and chloroandrostenone-17 used in these experiments.

[3] Melting points of these preparations were: androsterone, 181–182.5°; dehydroisoandrosterone, 138–139°; isoandrosterone, 172–173°; etiocholanone, 146–148°; and chloroandrostenone-17, 152–154°.

(Fig. 1). Distinct differences in the chromogenic capacities of the various 17-ketosteroids were found, but these divergences varied greatly with reaction concentration. Table 1 gives these results in detail. The following observations, confined to the four 17-ketosteroids, androsterone, dehydroisoandrosterone, isoandrosterone and 3-α-etiocholanone-17, may be noted:—

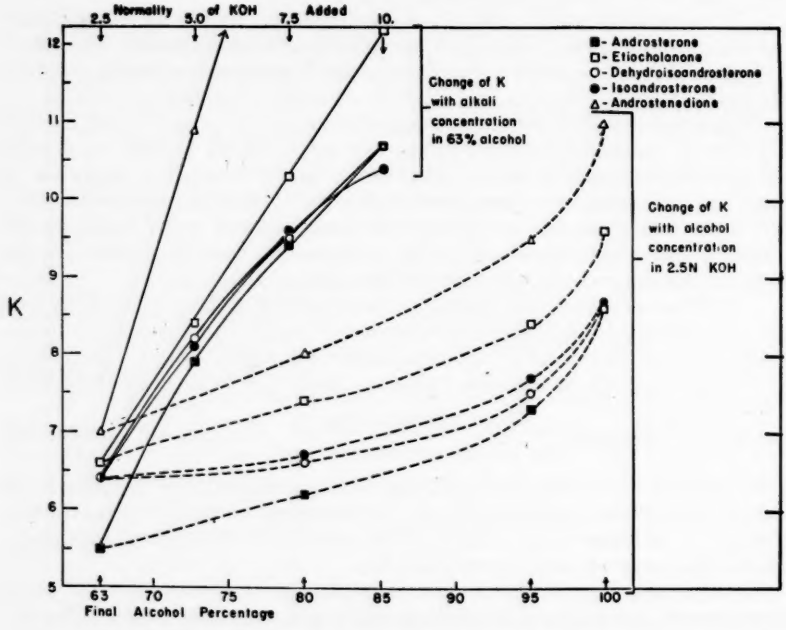

FIG. 1. Relations between chromogenic capacities of five 17-ketosteroids and concentration of alkali and alcohol. Continuous lines and upper abscissa show maximum K values as functions of alkali concentration. Broken lines and lower abscissa show maximum K values as functions of alcohol concentration.

1) Etiocholanone invariably had the highest chromogenic capacity. This divergence was greatest at high alkali concentrations, where etiocholanone reached 115% of the three other 17-ketosteroids.

2) Androsterone usually had the lowest chromogenic capacity. This divergence was greatest in the most dilute reaction mixture studied (2.5 N alkali in 63% alcohol). Here its K value was only 85% of the average of the others. Androsterone K became substantially the same as K for dehydroisoandrosterone and isoandrosterone with high concentrations of potassium hydroxide.

3) The two β-17-ketosteroids, isoandrosterone and dehydroisoandrosterone, had almost identical K values under most conditions, although in the strongest alkali isoandrosterone tended to produce less color. In contrast, the two α-ketosteroids, androsterone and etio-

cholanone, were widely separated throughout and were not brought to equivalent colors under any conditions. There was a difference even in the configuration of the curves relating their *K* values to concentration of alcohol or alkali (Fig. 1).

4) Increasing the alcohol concentration (using 2.5 N alkali) also caused androsterone color to approach that of the β-17-ketosteroids. In absolute alcohol these three were almost indistinguishable, but

TABLE I. COMPARISON OF K VALUES OBTAINED UNDER THE VARIOUS CONDITIONS BOTH AT MAXIMUM COLOR AND AT ONE SHORTER TIME INTERVAL. VOLUME OF REACTION MIXTURE IS 0.6 CC.

Conc. KOH Used	Final Alcohol Concentration	γ	Time	Androsterone			Dehydroisoandrosterone			Etiocholanone		Isoandrosterone			Androstenedione		
				K	%E	R	K	%E	R	K	R	K	%E	R	K	%E	R
N	%		Min.														
2.5	63	60	60	3.0	70	1.1	4.1	94	1.1	4.3	1.1	3.9	90	1.1	4.8	111	0.9
			180	5.5	83	1.0	6.4	97	1.0	6.6	1.0	6.4	96	1.0	7.0	106	0.9
5.0	63	40	45	6.0	81	1.4	6.8	92	1.5	7.4	1.4	6.7	91	1.4	9.5	128	1.1
			105	7.9	94	1.3	8.2	98	1.4	8.4	1.3	8.1	97	1.3	10.9	130	1.0
7.5	63	40	20	7.9	86	2.1	8.3	91	2.3	9.2	2.2	8.1	92	2.3		130	1.6
			60	9.4	91	2.2	9.5	92	2.4	10.3	2.1	9.6	93	2.4	15.5	150	1.3
10.0	63	30	20	10.7	88	2.8	10.7	88	3.0	12.2	2.6	10.4	85	2.9	18.0	147	1.2
2.5	80	50	90	5.4	78	1.2	6.4	92	1.3	7.0	1.3	6.0	87	1.3	7.9	113	1.1
			150	6.2	85	1.2	6.6	90	1.3	7.4	1.3	6.7	91	1.3	8.0	108	1.0
2.5	95	40	20	5.2	78	1.7	5.2	78	1.9	7.2	1.8	5.1	77	1.8	7.9	118	1.4
			50	7.3	86	1.8	7.5	90	1.9	8.4	1.9	7.7	91	1.8	9.5	110	1.4
2.5	100	30	20	6.9	76	2.2	7.6	84	2.2	9.1	2.2	7.4	81	2.0	10.4	114	1.6
			50	8.6	90	2.2	8.6	90	2.2	9.6	2.4	8.7	91	2.2	11.0	115	1.6
1.0	100	60	60	2.8	62	2.2	3.6	79	2.4	4.6	2.3	3.3	72	2.2	4.9	108	1.6
			180	5.2	84	2.3	5.8	94	2.5	6.1	2.5	5.4	86	2.6	6.8	110	1.8
4.0	100	30	15	11.2	91	2.6	11.2	91	2.7	12.5	2.8	11.2	91	2.7	14.9	119	1.9

%E is percentage of etiocholanone K.
R is the ratio of readings with 520 filter to 420 filter.
Maximum color was reached at the longer time interval given.

etiocholanone *K* was considerably higher (111%). No greater convergence of *K* values was effected by increasing alkali to 4.2 N in absolute alcohol.

5) The changes in relationships of *K* values with increasing alkali normalities were much the same in 100% alcohol as in 63%. This suggests that relationships would not be greatly different if alkali were varied at intermediate alcohol concentrations.

Androstenedione: This 3:17 ketosteroid had a higher chromogenic capacity than any of the 17-ketosteroids under all conditions. It was also the most sensitive to increasing alkali concentration, developing far more color than the others in strong potassium hydroxide (Table I).

Androsterone-17 and Chloroandrostenone-17: Some observations have been made on these ketosteroids, but maximum *K* values have been less well confirmed because of lack of material. In 63% alcohol *K* values for androsterone-17 were between those of etiocholanone and androsterone at all alkali concentrations. Chloroandrostenone-17 had

a lower chromogenic capacity than androsterone-17 in most cases. It does not appear that these two substances diverge from the average color values to any greater extent than has been observed for the other 17-ketosteroids studied.

Time Relations: The rate of reaction varied markedly with reagent concentration. Thus the time required for attainment of maximum color decreased sharply as reagent concentration was increased. For example, 180 minutes was required with 2.5 N potassium hydroxide in 63% alcohol, but with 10 N alkali the reaction was complete in 20 minutes (Table I).

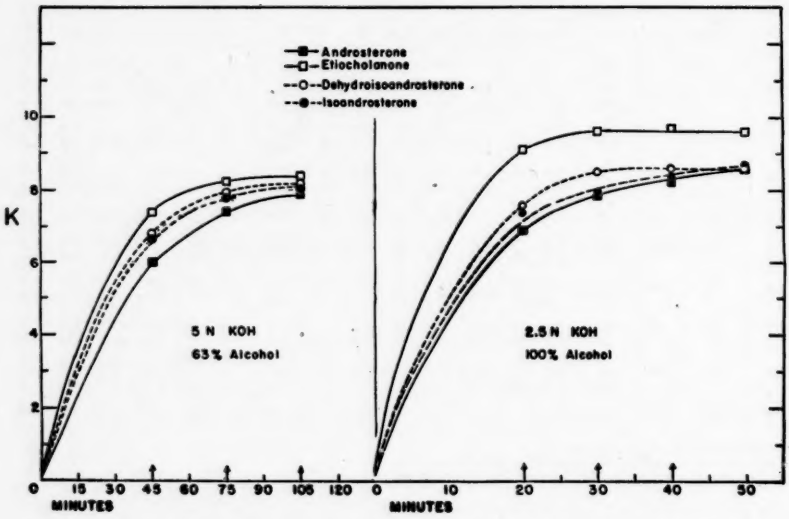

FIG. 2. Color-time curves of four 17-ketosteroids in two different reagent concentrations, showing convergence of *K* values at maximum color.

Chromogenic values of the various 17-ketosteroids were usually closer together at the time of maximum color than at shorter intervals. This is shown in Table I where maximum *K* values may be compared with those at one earlier time by noting the relation they bear to etiocholanone *K*. The explanation lies in differences in configuration of the individual color-time curves. Examples are given in Figure 2. While the actual maximum or constant color was attained at nearly the same time by all the 17-ketosteroids shown, there were marked differences in the per cent completion at earlier times. The curve for etiocholanone was always highest and it was the first to approach completion. Thus this curve had begun to flatten at a time when the others were still rising towards it. Similarly the androsterone curve continued rising towards that of dehydroisoandrosterone after the latter had become nearly flat. Hence the greatest similarity of *K*

values was found at maximum color development. These differences in configuration were most noticeable in absolute alcohol.

Absorption Spectra: Marked differences in the visual appearance of the color produced resulted from change in reaction conditions. Absorption spectra were therefore determined by reading the maximum color with five different filters from 420 to 540, setting the reagent blank at 100 in each case.

No important differences were found in the individual absorption curves of the 17-ketosteroids. The color developed with 2.5 N alkali in both 63% and 80% alcohol showed an absorption maximum at 490. As the normality of the alkali was increased, absorption became more nearly monochromatic at 520; the 520 constants rose while the 420 constants decreased. Hence there was an actual decrease in the amount of yellow reaction product formed by the pure 17-ketosteroids as the reaction mixture was made more alkaline. Increasing the alcohol percentage had similar effects but they were less marked. This advantage of higher alkali or alcohol concentration is somewhat offset by a simultaneous increase in reagent blank color and in experimental error.

Androstene-3:17-dione differed from the other 17-ketosteroids in showing a higher absorption at 420 relative to the 520 absorption. Callow *et al.* (1938) have shown that testosterone, a 3-ketosteroid, has a slight absorption maximum at 420; and that the absorption curve of androstenedione thus qualitatively resembles a mixture of a 3-ketosteroid and a 17-ketosteroid.

Mixtures: Saier *et al.* (1943) have shown that the chromogenic properties of androsterone were not altered by the presence of dehydroisoandrosterone in the same solution. The present authors have previously reported that the extent to which androsterone reacts was unaffected when it was added to the 17-ketosteroid fraction of a urine extract (Nathanson and Wilson, 1943). Possible influences of other pure 17-ketosteroids upon each other were sought in this work using various mixtures and several reaction conditions (Table II). The maximum absorption of each mixture was calculated for each set of conditions from the individual K values and the amount of each 17-ketosteroid present. A standard solution of dehydroisoandrosterone was included with every set of determinations to show any deviation from established K values. As can be seen in Table II, observed readings for all mixtures agreed closely with those calculated under all conditions. It is concluded that these four 17-ketosteroids are without effect on each other in the Zimmermann reaction.

Larger Volume of Reaction Mixture: The effect on maximum K values of varying the potassium hydroxide and alcohol concentrations has also been studied with the modified Oesting procedure where the reaction mixture has a volume of 14 cc. and is not further diluted before reading (Nathanson and Wilson, 1943). These results are summarized in Table III. The change in relative K values with increasing alkali

concentration was much the same as in the 0.6 cc. volume. In the most dilute reaction mixture (i.e., the 15% alkali (2.2 N) and 60% alcohol regularly employed), androsterone *K* was only 68% of *K* for the β-17-ketosteroids compared to 86% with the most dilute reaction mixture observed of 0.6 cc. volume (2.5 N alkali in 63% alcohol). The chromo-

TABLE II. OBSERVED AND CALCULATED READINGS OF MIXTURES OF 17-KETOSTEROIDS

Mixture No.	% Composition				L Readings at Maximum Color													
	A	D	E	I	2.5 N KOH			5 N KOH			7.5 N KOH			10 N KOH			2.5 N KOH in 80% Alcohol		
					Obs.	Calc.	%	Obs.	Calc.	%	Obs.	Calc.	%	Obs.	Calc.	%	Obs.	Calc.	%
1	45	5	45	5	.323	.316	102	.330	.326	101	.413	.408	101	.340	.341	99.7	.361	.362	100
2	29			71	.309	.314	98.5	.319	.316	101							.346	.346	100
3	29	53	18		.291	.293	99.3	.331	.324	102	.384	.382	100.2	.309	.320	96.5			
4	42		58		.274	.272	100.6	.312	.312	100	.370	.368	100.5	.297	.307	97.0			

A = Androsterone
D = Dehydroisoandrosterone
E = Etiocholanone
I = Isoandrosterone
Alcohol concentration is 63% except when otherwise noted.

TABLE III. COMPARISON OF K VALUES OBTAINED UNDER VARIOUS CONDITIONS BOTH AT MAXIMUM COLOR AND AT ONE SHORTER TIME INTERVAL. VOLUME OF REACTION MIXTURE IS 14 CC. (AFTER OESTING)

Conc. KOH Used	% Alc. Used	Mg. Ketosteroid	Time	Androsterone			Dehydroisoandrosterone			Etiocholanone		Isoandrosterone			Androstenedione		
				K	%E	R	K	%E	R	K	R	K	%E	R	K	%E	R
N			Hrs.														
2.2	60	0.5	3	0.48	62	0.73	0.68	89	0.76	0.76	0.75	0.70	92	0.75	0.86	113	0.60
			6	0.68	63	0.59	0.98	90	0.64	1.10	0.62	1.00	92	0.62	1.44	130	0.62
10	60	0.1 & 0.15	2	1.89	77	0.99	2.31	94	1.04	2.45	1.00	2.26	92	1.04	3.68	148	0.74
			4.5	2.27	89		2.58	100		2.57		2.47	96		4.15	160	
15	60	0.1 & 0.15	1	2.60	74	1.4	3.10	88	1.50	3.52	1.4	2.88	82	1.40	5.16	147	0.93
			2.5	3.52	86		3.77	92		4.09		3.74	92		6.38	156	
2.2	95	0.2 & 0.3	2	0.53	64	0.94	0.70	85	0.96	0.83	0.95	0.69	83	0.97	1.29	155	0.70
			5.5	0.95	68		1.40	100		1.40		1.16	83		2.23	160	

%E is percentage of etocholanone K.
R is ratio of 520 to 420 readings.
2.2 N KOH is the 15% solution regularly employed.

genic capacity of androsterone reached 94% of the β-17-ketosteroids when more concentrated alkali (15 N) was added. Etiocholanone *K* was about 10% above the β-17-ketosteroids at all alkali concentrations observed. These data confirm the previous contention that dilution or larger volume of the reaction mixture decrease the maximum androsterone color more than that of the other 17-ketosteroids (Nathanson and Wilson, 1943). Reaction mixtures of this type thus have the disadvantage of a wide spread of *K* values even at maximum color.

DISCUSSION

It is apparent that differences in chromogenic capacities may result in appreciable errors in assays of total 17-ketosteroid content. The magnitude of error will depend on the following factors:—

a) Reaction Conditions: Relationships among the 17-ketosteroids are highly dependent upon reaction conditions. Thus androsterone, dehydroisoandrosterone, and isoandrosterone approach equal chromogenic capacities as the time of reaction and the reagent concentration are increased, while etiocholanone and androstenedione may diverge. Equivalent colors are not developed by all 17-ketosteroids under any conditions.

b) Composition of the 17-ketosteroid Mixture: Some error will always be introduced in the assay of complex mixtures such as urine extracts by the use of any single 17-ketosteroid as a reference standard. Such errors may be minimized by employing conditions under which various 17-ketosteroids produce most nearly equivalent colors. In the present study, the greatest similarity was found at intermediate alkali concentrations, viz. 5 N potassium hydroxide in 63% alcohol (similar to Holtorff and Koch, 1940), or 2.5 N potassium hydroxide in absolute alcohol (similar to Callow *et al.*, 1938). This confirms the choice of conditions for assay previously suggested (Nathanson and Wilson, 1943).

When preparations of a single 17-ketosteroid are to be assayed, as in isolation studies, the most accurate procedure would be to use a crystalline sample of the same 17-ketosteroid as a reference standard. Reaction conditions would then be of little importance. However, if one 17-ketosteroid were always used as a reference standard in this type of experiment, appreciable errors could appear under certain conditions. For example, in a reagent concentration of 2.5 N alkali and 63% alcohol, a mixture consisting chiefly of etiocholanone would be overestimated by about 18% against an androsterone standard, whereas a solution containing mainly androsterone would be underestimated by approximately 15% if dehydroisoandrosterone were the standard.

c) Reference Standard: A reference standard for general use, as with urinary extracts, should be suitable under most reaction conditions. Dehydroisoandrosterone is usually intermediate in the chromogenic capacities of the different 17-ketosteroids under varying concentrations. Therefore it appears most satisfactory as a reference standard in order to minimize errors in assay. Androsterone, which is now the common standard, is obviously the least satisfactory substance, especially in dilute reaction mixtures. Although the color-time curve of etiocholanone bears a striking similarity to those of urinary extracts, particularly in absolute alcohol, its chromogenic capacity is greater than that of the other common 17-ketosteroids under any conditions of reaction.

The time of reaction also affects the accuracy of 17-ketosteroid assays. Under most reaction conditions various 17-ketosteroids give most nearly equivalent colors when maximum absorption has been reached. At earlier reading times differences in initial rates of reaction of individual 17-ketosteroids result in a wide range of color values.

These findings support the authors' previous conclusion that the time at which both the crystalline 17-ketosteroid reference standard and urine extracts reach maximum and constant color is the optimum reading time (Nathanson and Wilson, 1943).

In studying the effect of reagent concentration it was logical to make observations at the times of maximum absorption in order that comparison might be made between reactions which had all proceeded to 100% completion. When a reading time of 1 hour is used at all concentrations, as in the work of Hansen *et al.* (1943), the reaction is only partially complete in the weaker alkali normalities. Since the average rate of reaction of the various 17-ketosteroids is different at each concentration, each such observation represents a different per cent completion. Thus a true picture of chromogenic capacities as functions of reagent concentration is not obtained.

SUMMARY

A study has been made of the relative chromogenic properties of seven crystalline 17-ketosteroids in the Zimmermann reaction under varying reaction conditions. Maintaining other factors constant, the alcohol concentration was varied from 63% to 100% and the potassium hydroxide from 2.5 N to 10 N. In order that true chromogenic capacities could be compared, observations were made at the time of maximum color under each set of conditions.

Relationships among the 17-ketosteroids depend upon reaction conditions. Equivalent colors were not produced by all 17-ketosteroids under any conditions. The range of color values was widest in dilute alkali, where androsterone produced the least color, but in very strong alkali etiocholanone and androstenedione diverged. In general the chromogenic properties of the common 17-ketosteroids approached equality as either the alcohol or alkali concentration was increased. The greatest similarity was found at intermediate alkali concentrations, i.e., 5 N potassium hydroxide in 63% alcohol, or 2.5 N potassium hydroxide in absolute alcohol. Even in these instances etiocholanone was not identical with the others.

Differences in chromogenic capacities may cause appreciable errors in assays of 17-ketosteroid content, but these errors may be minimized by suitable choice of reaction conditions and of reference standard.

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NOTES AND COMMENTS

METABOLISM OF THE STEROID HORMONES: THE CONVERSION OF Δ^5-ANDROSTENEDIOL-3(β),17(α) TO DEHYDROISOANDROSTERONE

The presence of Δ^5-androstenediol-3(β),17(α) has been demonstrated in the urine of a boy suffering from an adrenal cortical cancer (Hirschmann and Hirschmann, 1945) and in the urine of a woman with an adrenal cortical cancer (Schiller, Miller, and Dorfman, 1945). It has been further demonstrated that the administration of Δ^5-androstenediol-3(β),17(α) to man gives rise to an increased urinary excretion of 17-ketosteroids (Frame, Fleischmann and Wilkins, 1944). This study is concerned with the conversion of Δ^5-androstenediol-3(β),17(α) into dehydroisoandrosterone.

Each of three normal adult male guinea pigs received 150 mg. of Δ^5-androstenediol-3(β),17(α) diacetate[1] daily for four days. The steroid was dissolved in sesame oil and injected subcutaneously. A total dose of 1,800 mg. of the diacetate was administered. Urine was collected for ten days during the pre-injection period, during the injection period, and for five days after the injections were discontinued. The urines were acidified with 10% concentrated hydrochloric acid by volume, refluxed for 10 minutes, and extracted three times with benzene (1 part benzene to 4 parts urine). The neutral fraction was further fractionated with the Girard-Sandulesco reagent *T* into the ketonic and nonketonic fractions.

The ketonic neutral fraction prepared from the urine collected during the injection and post-injection periods weighed 114.5 mg. After precipitation with digitonin from 90% ethanol solution, 12.5 mg. of crystalline digitonin precipitable ketonic neutral material was obtained. Recrystallization of this material from aqueous methanol yielded 7.2 mg. of a substance with a melting point of 143°–148°[2]. Further recrystallization from aqueous methanol yielded crystals melting at 146°–148.5°. When mixed with an authentic sample of dehydroisoandrosterone, melting point 149°–150°C., the melting point was 147.5°–150°C. An acetate was prepared which melted at 159°–160° and when mixed with an authentic sample of dehydroisoandrosterone acetate, M.P. 163°–164.5°C., the mixture melted at 159°–163°.

Normal male guinea pigs excreted 0.77 mg. per day of ketonic neutral material, as compared to 4.2 mg. per day during the nine days representing the injection and immediate post-injection periods. The ketonic neutral fraction prepared from the pre-injection urine yielded no digitonin precipitable material.

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[1] We are indebted to Ciba Pharmaceutical Products, Inc., for a generous supply of Δ^5-androstenediol-3(β),17(α).

[2] The melting points are uncorrected and were determined with the Fisher-Johns apparatus.

SUMMARY

Dehydroisoandrosterone has been isolated from the urine of male guinea pigs following the administration of Δ^5-androstenediol-3(β),17(α).

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EFFECTS OF THYROID FEEDING ON OVARIAN DEVELOPMENT IN THE RAT

Reports of the effects of thyroid feeding on the ovaries of rats are conflicting. Herring (1917) and Korenchevsky, Hall and Clapham (1943) observed ovarian hypertrophy subsequent to thyroid feeding, while Kamiovski (1938) and Drill, Overman and Leathem (1943) reported a loss of ovarian weight. The work of Drill *et al.* (1943) indicates that these differences may be due in part to the vitamin content of the rations employed. It was observed that although thyroid feeding (100 mg. per day) caused anestrus and loss of body and ovarian weight in the adult rat, estrus cycles remained regular and loss of body and ovarian weight were prevented if thiamine were also administered or if the diet were supplemented with yeast. The anestrus and loss of ovarian weight were presumably due to thiamine deficiency resulting from the increased requirement for this vitamin in the thyroid fed rat and manifesting itself in impaired secretion of pituitary gonadotropins. When sufficient quantities of yeast were administered to prevent a loss of body weight in adult rats, estrus cycles remained regular and loss of ovarian weight was prevented. In the present experiment desiccated U.S.P. thyroid was fed to immature rats receiving sufficient quantities of yeast to maintain growth, and the effects of thyroid feeding on ovarian weight and histology determined.

Experimental: The basal ration consisted of sucrose 61.5, Vitamin Test Casein[1] 22.0, yeast[2] 12.0 and Sure's Salt Mixture No. 1[3] 4.5. Desiccated thyroid[4] was incorporated in the above ration at levels of 0.0, 0.5 and 1.0 per cent replacing equal quantities of sucrose. Daily supplements of 500 mg. corn oil (Mazola) and an A-D concentrate[5] equivalent to 50 U.S.P. units of

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[1] General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Brewers' Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram according to the manufacturer contained the following vitamin potencies: thiamin 600 micrograms, riboflavin 75 micrograms, pyridoxine 100–125 micrograms, pantothenic acid 420–560 micrograms, nicotinic acid 350–500 micrograms and biotin 0.2–0.5 micrograms.

[3] Sure, Barnett. 22: 499. 1941.

[4] U.S.P. Desiccated Thyroid, Armour & Co., Los Angeles, California.

[5] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

vitamin A and 5 U.S.P. units of vitamin D were also administered. Forty-two female rats of the Long-Evans strain were employed in the present experiment. Animals were weaned at twenty-one to twenty-three days of age at an average weight of 41.2 grams, and litter mates were divided as far as possible among the following three groups: (1) twelve rats maintained on the thyroid free ration indicated above (2) eighteen animals on a similar ration but containing 0.5 per cent desiccated thyroid and (3) twelve rats on a similar diet but containing 1.0 per cent desiccated thyroid. The rations were administered *ad lib.*, and feeding was continued for sixty days.

Results: Growth was retarded in all thyroid fed rats, but animals grew consistently on the rations, averaging after sixty days of feeding 168.2 g. ± 7.3,[6] 141.3 g. ± 12.6[6] and 118.6 g. ± 9.1[6] g. for the 0.0, 0.5 and 1.0 per cent thyroid series respectively. Subsequent to the fortieth day of feeding vaginal smears were taken daily of all rats. Animals receiving thyroid exhibited constant leucocytic smears in contrast to the regular estrus cycles of thyroid free controls. Eleven rats (61.1%) and 5 rats (41.7%) of the groups receiving 0.5 and 1.0 per cent thyroid respectively survived the experimental period of sixty days at which time surviving animals were autopsied, ovaries fixed in formol, and sections prepared and stained with hematoxylin and eosin.[7]

A marked inhibition of ovarian development was observed both grossly and histologically in thyroid fed rats. Ovarian weights averaged 20.0 and 21.3 mgms respectively for animals in the 0.5 and 1.0 per cent thyroid series in contrast to an average weight of 42.8 mgms. in thyroid free controls. Histologically the ovaries resembled those of immature rats twenty to thirty days of age.[8] No mature follicles or corpora lutea were present in any of the thyroid fed rats; immature follicles were numerous and many young follicles were observed undergoing atresia. Moderate degrees of edema were noted in the medullary portion, and there was evidence of hemostasis in the medullary blood vessels. The germinal epithelium appeared normal. No significant differences were noted between animals on the 0.5 and 1.0 per cent thyroid levels. In rats maintained on thyroid free rations ovaries were normal throughout.

Discussion: Failure of ovarian development in thyroid fed rats reported above is in marked contrast to the findings of Drill *et al.* (1943) who observed normal ovarian function in adult thyroid fed rats when sufficient quantities of yeast were administered to prevent a loss in body weight. Although growth was retarded in the present series, thyroid fed rats made consistent gains in body weight during the experimental period indicating that nutritional requirements for growth were being met. Ovaries nevertheless remained infantile and did not differ significantly in weight or histology from those observed in twenty-one day old animals of this strain. These differences may be due to the respective age of experimental animals at the start of thyroid feeding, a suggestion in accord with the findings of Bodansky and Duff (1936). The infantile ovaries of the thyroid fed rats suggest that failure

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[6] Including standard error of the mean calculated as follows: $\sqrt{\epsilon(d^2/n)}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[7] Average survival time of decendents was 41.3 days.

[8] We are indebted to Dr. Boris Krichesky, Department of Zoology, University of California at Los Angeles, for his assistance in the examination and interpretation of histological material.

of ovarian development was due to impaired secretion of pituitary gonadotropins, although refractoriness of ovarian tissue to gonadotropic stimulation or an increased ovarian threshold to pituitary gonadotropins might account for the same results. No luteinization was observed in the present series as reported by Weichert and Boyd (1933) and Koger and Turner (1943) following thyroid feeding in the rat, a difference that may be due to the level of thyroid fed, the age of experimental animals at the start of thyroid feeding, the composition of the basal diet or the strain of rat employed.

Summary: Female rats were raised to maturity on diets containing 0.0, 0.5 and 1.0 per cent desiccated thyroid. A marked inhibition of ovarian development was observed in thyroid fed rats, ovaries remaining infantile both in weight and histological appearance.

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ESTIMATION OF MICROGRAM QUANTITIES OF IODINE WITH THE PHOTOELECTRIC COLORIMETER

Recently Talbot and co-workers (1944) and Salter and co-workers (1944) have published methods for iodine determination in biological materials utilizing photoelectric colorimeters for final estimation, Talbot measuring the intensity of the starch-iodine color and Salter the catalytic effect of iodide on the ceric-arsenite reaction. In 1940–41 we had briefly utilized both these principles and are now presenting a short summary of our data and experimental conditions in substantiation of their general utility. The procedure for blood iodine determination then in use in this laboratory was a modification (Strickler and Wilson, 1938; Grauer and Saier, 1939) of the method of Stimmel and McCullagh (1936), which uses distillation to remove the iodine from the salts present after alkali ashing. We always made up series of standards containing the same constituents (2 cc. 2% sodium bisulfite, 0.8 cc. 3% sulfuric acid, 15 cc. water) as the absorption solution in the distillate receivers, plus known amounts of potassium iodide or iodate solutions. Potassium iodide could be added either before boiling to expel sulfur dioxide or after adjusting with potassium hydroxide (4 cc. 1:100 saturated) to alkalinity, but potassium iodate could not be added after sulfur dioxide expulsion if the ceric-arsenite reaction was to be used, since we found practically no catalytic effect then, though the arsenious acid was added just before the ceric ammonium sulfate.

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A few blood samples which had already been run with titrimetric estimation of the results were re-run and the results estimated by one (or both, in one case) of the photoelectric procedures. The data are shown in Table I.

TABLE I

Patient	BMR %	Micrograms per 100 cc. blood by titration*	by starch-iodine colorimetry	by ceric-arsenite catalysis
K	+9; +6; +18	4.4; 4.6	4.2	3.6
R	+29	6.9	—	6.6
G		2.6	—	2.4
M		4.0	—	4.5
B		2.3	—	2.6
D	+40	8.9	—	10.9
H		3.4	3.4	—

* The normal range in Pittsburgh was approximately 2 to 4μg. per 100 cc. blood.

The values are within the same range, which we feel is important, since it indicates that the material from these specimens which reacts in the Winkler reaction and that which catalyzes the ceric-arsenite reaction is indeed iodine.

For the ceric-arsenite reaction, we used the procedure of Chaney (1940) except that the volume had to be five times as large to fill the cells of the Fisher[1] colorimeter employed, and that we made readings only once, after forty minutes reaction time at 30°. The colorimeter null blank was merely distilled water. The per cent transmittance was plotted against the amount of iodine added to the standards, and the unknowns and blanks evaluated from this curve. For blood iodine estimations, the distillate was in some cases made up to 50 cc., from which aliquots were taken and diluted, the proper extra amounts of absorption solution also being added. The average result calculated from estimation in the usual manner of these aliquots was taken for report. For blank determinations, known amounts of potassium iodide were added to some of the aliquots, this amount of iodine being subtracted after estimation. For example, a 1/10 aliquot to which 0.051 μg. had been added gave 0.057, net 0.006 or a blank of 0.060 μg. A ½ aliquot to which 0.051 μg. had been added gave 0.083, net 0.032 or a blank of 0.064 μg. and so on.

For the colorimetric estimation of the starch-iodine color we used the micro-attachment of the above colorimeter, with Corning Filter No. 348, 3 mm. (cf. Müller and McKenna, 1936). Both standards and unknowns were boiled to 5 cc. and cooled, then after the addition of 1 drop of very dilute methyl orange, 3% sulfuric acid was added to adjust to acid, plus 0.1 cc. excess. Bromine was blown in from a brominator. After ten minutes at room temperature, the tubes were placed in a boiling water bath till expulsion of visible bromine, then boiled to 3 cc. over a micro-burner. After cooling in ice, 0.2 cc. starch (Nichols, 1929) and 0.2 cc. 0.8% potassium iodide were added, the material transferred to a chilled cell, made up to 4 cc. with iced water and read in the colorimeter within three to five minutes. No attempt was made to combine standard data from different days (Sendroy,

[1] Fisher Catalog No. 7-089, battery operated.

1939). Titrimetric estimation with 0.001 N thiosulfate had been carried out since 1937 using three or four point standard curves for evaluation of unknowns. These were treated essentially as above. The final volume at the time of the comparisons shown was 2 cc., and the additions of starch and potassium iodide were but 0.1 cc. of each.

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